(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
17 July 2003 (17.07.2003)

PCT

(10) International Publication Number
WO 03/057897 A2

(51) International Patent Classification[7]: C12P 21/00, C12N 15/67, A61K 48/00, 38/16

(21) International Application Number: PCT/IB03/00299

(22) International Filing Date: 7 January 2003 (07.01.2003)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
0200250.9 7 January 2002 (07.01.2002) GB
0209013.2 19 April 2002 (19.04.2002) GB

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(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PT, SE, SI, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *without international search report and to be republished upon receipt of that report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 03/057897 A2

(54) Title: RECOMBINANT PROTEIN EXPRESSION

(57) Abstract: There are provided methods for the expression of a recombinant protein of interest, said methods comprising, in additional to various additional steps: a) culturing a host cell which expresses: i) one or more genes encoding the recombinant protein(s) of interest;ii) at least two genes encoding proteins selected from the group consisting of the chaperone proteins GroEL, GRoES, Dnak, DnaJ, GRpe, ClpB and their homologs (for example, Hsp104, Ydj1 and Ssal in yeast); under conditions suitable for protein expression; and separating said recombinant protein of interest from the host cell culture. Also provided are methods for increasing the degree of refolding of a recombinant protein of interest by ading a composition containing a chaperone protein to a preparation of the recombinant protein of interest in vitro.

Recombinant Protein Expression

The invention relates to methods for increasing the yield of folded recombinant protein in host cells.

All publications, patents and patent applications cited herein are incorporated in full by reference.

The overproduction of recombinant proteins in cellular systems frequently results in misfolding of these proteins. The fates of the misfolded recombinant proteins differ. They may refold to the native state or be degraded by the proteolytic machinery of the cell or be deposited into biologically inactive large aggregates known as 'inclusion bodies'.

The folding of proteins and the refolding of misfolded soluble and aggregated proteins is known to be mediated by a network of evolutionarily conserved protein molecules called chaperones (Hartl, F.U., *Nature,* 381, 571-580, (1996); Horwich, A.L., Brooks Low K., Fenton, W.A., Hirshfield, I.N. & Furtak, K., *Cell* 74, 909-917 (1993); Ellis, R.J. & Hemmingsen, S.M., *TiBS,* 14, 339-342, (1989); Bukau, B., Hesterkamp, T. & Luirink, J., *Trends Cell Biol.,* 6, 480-486, (1996); Bukau, B., Deuerling, E., Pfund, C. & Craig, E.A., *Cell,* 101, 119-122, (2000)). Major chaperones include members of evolutionarily conserved protein families, including the Hsp60 family (which includes the bacterial chaperone GroEL), the Hsp70 family (which includes the bacterial chaperone DnaK), the Hsp100 family (which includes the bacterial chaperone ClpB), the Hsp90 family (which includes the bacterial chaperone HtpG), the bacterial Trigger factor family, and the small HSPs (which includes the bacterial proteins IbpA and IbpB).

Bacterial systems like the gram-negative bacterium *Escherichia coli* are a popular choice for the production of recombinant proteins. In *E. coli*, it is known that the DnaK and GroEL/ES chaperone systems assist the *de novo* folding of proteins (Hartl, F.U., *Nature,* 381, 571-580, (1996); Ewalt, K.L., Hendrick, J.P., Houry, W.A. & Hartl, F.U. *Cell* 90, 491-500 (1997); Bukau, B., Deuerling, E., Pfund, C. & Craig, E.A., *Cell,* 101, 119-122, (2000); Teter, S.A. et al., *Cell,* 97, 755-765, (1999); Bukau, B. & Horwich, A.L, *Cell,* 92, 351-366, (1998); Deuerling, E., Schulze-Specking, A., Tomoyasu, T., Mogk, A. & Bukau, B. *Nature* 400, 693-696 (1999)).

Furthermore, DnaK and its co-chaperones DnaJ and GrpE are presently considered to form the most efficient chaperone system for preventing the aggregation of misfolded proteins

(Mogk, A. et al., *EMBO J.,* 18, 6934-6949, (1999); Tomoyasu, T., Mogk, A., Langen, H., Goloubinoff, P. & Bukau, B., *Mol, Microbiol.,* 40, 397-413, (2001); Gragerov, A. et al., *Proc. Natl. Acad. Sci. U.S.A.* 89, 10341-10344 (1992)). Increased levels of GroEL and its co-chaperone GroES have been shown to prevent the heat induced aggregation of proteins in cells deficient of other major chaperones (Tomoyasu, T., Mogk, A., Langen, H., Goloubinoff, P. & Bukau, B., *Mol, Microbiol.,* 40, 397-413, (2001); Gragerov, A. et al., *Proc. Natl. Acad. Sci. U.S.A.* 89, 10341-10344 (1992)).

Moreover, the disaggregation of protein aggregates in *E. coli* using chaperones has been proven for many different cellular proteins *in vivo* (Mogk, A. et al., *EMBO J.,* 18, 6934-6949, (1999)), as well as *in vitro* using thermolabile malate dehydrogenase (MDH) as a reporter enzyme (Goloubinoff, P., Mogk, A., Peres Ben Zvi, A., Tomoyasu, T. & Bukau, B., *Proc. Natl. Acad. Sci., USA* 96, 13732-13737, (1999)). Protein disaggregation is achieved by a bi-chaperone system, consisting of ClpB and the DnaK system. Large aggregates of MDH could be resolubilised *in vitro* and MDH was refolded afterwards into its native structure. Importantly, only the combination of both chaperones is active in resolubilisation and refolding of aggregated proteins. A recent publication showed that the resolubilisation of recombinant proteins from aggregates *in vivo* is possible. In these experiments, protein aggregates were generated by temperature upshift, and the solubilisation and refolding of these proteins was measured in the presence of protein synthesis inhibitors to ensure that only the pre-existing aggregated proteins were monitored. Molecular chaperones were able to resolve the aggregates under these conditions.

Previous studies also indicate that the solubility and yield of recombinant proteins could be enhanced by the overproduction of chaperones. Co-overproduction of GroEL/GroES enhanced the solubility of several recombinant proteins synthesised in *E. coli* (human ORP150, human lysozyme, $p50^{csk}$ protein tyrosine kinase, phosphomannose isomerase, artificial fusion protein PreS2-S′-ß-galactosidase) (Amrein, K.E. et al., *Proc. Natl. Acad. Sci., USA* 92, 1048-1052 (1995); Nishihara, K., Kanemori, M., Yanagi, H. & Yura, T., *Appl. Environ. Microbiol.,* 66, 884-889 (2000); Thomas, J.G. & Baneyx, F., *Mol. Microbiol.* 21, 1185-1196 (1996); Proudfoot, A.E., Goffin, L., Payton, M.A., Wells, T.N. & Bernard, A.R. *Biochem J* 318, 437-442. (1996); Dale, G.E., Schönfeld, H.J., Langen, H. & Stieger, M., *Protein Engineering,* 7, 925-931 (1994)). The overproduction of the DnaK system together with recombinant target proteins elevates the solubility of endostatin,

human ORP150, transglutaminase and the fusion protein PreS2-S′-ß-galactosidase (Nishihara, K., Kanemori, M., Yanagi, H. & Yura, T., *Appl. Environ. Microbiol.*, 66, 884-889, (2000); Thomas, J.G. & Baneyx, F., *J Biol Chem* 271, 11141-11147 (1996); Yokoyama, K., Kikuchi, Y. & Yasueda, H., *Biosci. Biotechnol. Biochem.* 62, 1205-1210 (1998)).

So far, no systematic approach has been made, to analyse whether the combination of all three chaperones systems (DnaK, DnaJ GrpE; GroES, GroEL and ClpB) expressed together with target genes in *E. coli* cells enhances solubility of recombinant proteins. Furthermore, none of the above-described studies allows the widespread optimisation of expression systems that is required to improve yields of soluble proteins on a general level. For example, each of the prior investigations focused on only one or a very small number of target proteins. These investigations also focused on the use of only one or two combined chaperone systems. In addition, none of these investigations addressed the issue of the importance of the ratio of the chaperones to one another and to the recombinant target protein. The previous studies therefore did not provide any understanding of the relationship between different chaperone proteins with respect to the folding/refolding of recombinant target proteins.

Accordingly, there remains a great need in the art for a general method to improve the yield of soluble recombinant protein in a given expression system. Such a method would allow the optimisation of expression systems to give maximal yields of soluble target proteins, and be of obvious industrial and commercial benefit.

The present invention is based upon the systematic engineering of cells for the controlled co-overexpression of different combinations of chaperone genes and target genes. In addition, it was investigated whether in vivo disaggregation and refolding of recombinant proteins from aggregates/inclusion bodies could be stimulated by enhanced levels of chaperones when the production of the target protein is stopped. As a result, the invention provides novel methods of optimising a given expression system in order to achieve higher yields of the desired soluble recombinant protein.

According a first aspect of the present invention, there is provided a method for the expression of a recombinant protein of interest, said method comprising:

a) culturing a host cell which expresses:

i) one or more genes encoding one or more recombinant protein(s) of interest;

ii) at least two genes encoding proteins selected from the group consisting of the chaperone proteins GroEL, GroES, DnaK, DnaJ, GrpE, ClpB and their homologs (for example, Hsp104, Ydj1 and Ssa1 in yeast); under conditions suitable for protein expression; and

b) separating said recombinant protein of interest from the host cell culture.

Through the recombinant engineering of host cells in this manner, the invention provides novel methods for producing a recombinant protein of interest, which have been found to lead to significant improvements in the levels of protein produced in the system. The mechanism is thought to be through increasing the folding rates of particular proteins using the co-expression of particular chaperones in controlled amounts. Using this system, very high yields of the desired soluble recombinant proteins of interest can be obtained.

Any recombinant protein of interest may be produced using the system of the invention. Preferred examples of proteins of interest will be apparent to the skilled reader. Particularly preferred recombinant proteins are those for which it is desirable to produce a large amount, and those of commercial interest.

Furthermore, the invention is readily applicable to a wide range of known expression systems by alterations in the cell culture techniques employed. For example, anaerobic fermenter-based cell culture would be appropriate for the culture of obligate anaerobes, whereas standard aerobic cell culture techniques would be appropriate for obligate aerobes. The nutrient composition of the culture medium may also be varied in accordance with the chosen expression system. The most suitable method of cell culture for a given expression system will be readily apparent to the skilled man.

Preferably, the genes selected in step a) ii) include DnaK, DnaJ and GrpE or homologs thereof, and may additionally include ClpB or a homolog thereof.

In another preferred aspect of the invention, the genes selected in step a) ii) include GroES and GroEL or homologs thereof.

More preferably, the genes selected in step a) ii) include the DnaK, DnaJ, GrpE, ClpB, GroES and GroEL genes or homologs thereof.

The above combinations of chaperone proteins have been found to be particularly suitable for use in the methods according to the invention.

According to a further embodiment of the first aspect of the present invention, there is provided a method for the expression of a recombinant protein of interest, said method comprising:

a) culturing under conditions suitable for protein expression a host cell which expresses:

i) one or more genes encoding one or more recombinant protein(s) of interest;

ii) one or more genes encoding proteins selected from the group consisting of the chaperone proteins GroEL, GroES, DnaK, DnaJ, GrpE, ClpB and their homologs (for example, Hsp104, Ydj1 and Ssa1 in yeast);

iii) one or more genes encoding proteins selected from the group consisting of the small heatshock proteins of the IbpA family and/or the IbpB family and/or their homologs; and

b) separating said recombinant protein of interest from the host cell culture.

The inclusion of a small heatshock protein of the IbpA family and/or the IbpB family with one or more of the chaperone proteins GroEL, GroES, DnaK, DnaJ, GrpE, ClpB in a host cell with a gene encoding a protein of interest has been shown to bestow significant beneficial effects on the level of expression of the recombinant protein.

For the purposes of this patent specification, two genes or proteins are said to be 'homologs' if one of the molecules has a high enough degree of sequence identity or similarity to the sequence of the other molecule to infer that the molecules have an equivalent function. 'Identity' indicates that at any particular position in the aligned sequences, the amino acid or nucleic acid residue is identical between the sequences. 'Similarity' indicates that, at any particular position in the aligned sequences, the amino acid residue or nucleic acid residue is of a similar type between the sequences. Degrees of identity and similarity can be readily calculated (Computational Molecular Biology, Lesk A.M., ed., Oxford University Press, New York, 1988; Biocomputing, Informatics and Genome Projects, Smith, D.W., ed., academic Press, New York, 1993; Computer Analysis of Sequence Data, Part 1, Griffin, A.M., and Griffin, H.G., eds., Humana Press, New

Jersey, 1994; Sequence Analysis in Molecular Biology, von Heinje, G., Academic Press, New Jersey, 1987; and Sequence Analysis Primer, Gribskov, M. and Devereux, J., eds., M Stockton Press, New York, 1991).

The chaperone proteins for use in the invention therefore include natural biological variants (for example, allelic variants or geographical variations within the species from which the genes are derived) and mutants (such as mutants containing nucleic acid residue substitutions, insertions or deletions) of the genes. For the purposes of this application, greater than 40% identity between two polypeptides is considered to be an indication of functional equivalence. Preferred polypeptides have degrees of identity of greater than 70%, 80%, 90%, 95%, 98% or 99%, respectively. It is expected that any protein that functions effectively as a chaperone, or as part of a chaperone system, within the host cells of the expression system will be of value in the described methods.

Preferably, the levels of the respective chaperone proteins are controlled in conjunction with the methods described above. Preferably, the levels of chaperone proteins are controlled by expressing the genes encoding the respective chaperone proteins from different promoters. Preferably, a selection or all of the promoters used are inducible. Different promoters may have different strengths and may respond to the same induction agent with different kinetics or be responsive to a different induction agent, allowing independent control of the expression level of each chaperone protein. Suitable promoters will be apparent to those of skill in the art and examples are given in standard textbooks, including Sambrook et al., 2001 (Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY); Ausubel et al., 1987-1995 (Current Protocols in Molecular Biology, Greene Publications and Wiley Interscience, New York, NY). Examples of suitable promoters include IPTG-regulated promoters, such as the PA11 and lac-O1 promoters (see Tomoyasu, 2001).

Alternatively, or in addition, the respective chaperone proteins are expressed using expression systems of different strength. Examples of different expression systems will be clear to those of skill in the art; discussion of such systems may be found in standard textbooks, including Sambrook et al., 2001 (*supra*) and Ausubel et al., (*supra*). For example, the plasmid vector of the expression system may be a high copy number or low copy number plasmid. For instance, examples of *E.coli* compatible low copy number plasmids include pSC101 and p15A ori.

Preferably, the chaperone proteins are over-expressed relative to the expression levels that occur naturally in non-recombinant cells.

Similarly, the invention provides for the levels of the chaperone proteins relative to the recombinant protein(s) of interest to be controlled by expressing the genes encoding the respective proteins from different promoters, for the reasons described above. For example, in a system that utilises an IPTG-inducible promoter for expression of chaperone proteins, an arabinose-inducible promoter may be used to control expression of the recombinant protein of interest. In addition, the expression of the chaperones and of the recombinant proteins(s) can be controlled using different polymerases.

In a second aspect, the invention also provides methods comprising the use of a block in protein synthesis during the culturing steps a) described above. Preferably, the block in protein synthesis is imposed by addition of an effective amount of a protein synthesis inhibitor to the culture system, once a desired level of recombinant protein of interest has accumulated. More preferably, the chosen protein synthesis inhibitor is chloramphenicol, tetracycline, gentamycin or streptomycin. In order to ensure that protein synthesis is adequately inhibited, an effective amount of a protein synthesis inhibitor should be added. Details of effective amounts of protein synthesis inhibitor will be apparent to the skilled reader and are noted in standard textbooks. For example, for use in prokaryotic host cell systems, 200μg/mL chloramphenicol is effective to inhibit protein synthesis.

Any other method that inhibits protein synthesis may also be of value for use with the methods of the invention. This includes the use of mutant strains that are conditionally defective in protein synthesis, for example because of the temperature sensitivity of an enzyme involved in plasmid or host cell DNA replication or in target gene and host gene transcription or in protein translation. The imposition of such a block in protein synthesis has been found to lead to significant increases in the level of recombinant protein that is generated in the system of the invention.

Alternatively, or in addition, the invention also provides for the use of a reduction in gene transcription, by removal of any agents that are effective to induce recombinant protein expression (such as IPTG for Lac repressor controlled genes), once a desired level of recombinant protein of interest has accumulated. Alternatively, a reduction of construct transcription could be achieved via the addition of a transcription blocking compound (such as glucose for catabolite repressable genes).

This aspect of the invention thus provides a method for the expression of a recombinant protein of interest, said method comprising:

a) culturing a host cell which expresses:

i) one or more genes encoding one or more recombinant protein(s) of interest;

ii) one or more genes encoding one or more proteins selected from the group consisting of the chaperone proteins GroEL, GroES, DnaK, DnaJ, GrpE, ClpB and their homologs (for example, Hsp104, Ydj1 and Ssa1 in yeast); under conditions suitable for protein expression;

b) imposing a block in protein synthesis, for example by addition of an effective amount of a protein synthesis inhibitor to the culture system, once a desired level of recombinant protein of interest has accumulated; and

c) separating said recombinant protein of interest from the host cell culture.

Also provided is a method for the expression of a recombinant protein of interest, said method comprising:

a) culturing a host cell which expresses:

i) one or more genes encoding one or more recombinant protein(s) of interest;

ii) one or more genes encoding one or more proteins selected from the group consisting of the chaperone proteins GroEL, GroES, DnaK, DnaJ, GrpE, ClpB and their homologs (for example, Hsp104, Ydj1 and Ssa1 in yeast); under conditions suitable for protein expression;

b) imposing a reduction in gene transcription, for example by removal of any agents that are effective to induce recombinant protein expression (such as IPTG for Lac repressor controlled genes), or via the addition of a transcription blocking compound (such as glucose for catabolite repressable genes), once a desired level of recombinant protein of interest has accumulated; and

c) separating said recombinant protein of interest from the host cell culture.

One or more genes encoding proteins selected from the group consisting of the small heatshock proteins of the IbpA family and/or the IbpB family and/or their homologs may

also be included in the host cell. The inclusion of such proteins in conjunction with the imposition of a reduction in gene transcription or the imposition of a block in protien synthesis.

Preferably, a combination of chaperone proteins is expressed as described above.

Preferably, the chaperone proteins are expressed under a different promoter to that used to control expression of the recombinant protein of interest.

Preferably, the chosen protein synthesis inhibitor is chloramphenicol, tetracycline, gentamycin or streptomycin.

Preferably, in the methods of the above-described aspects of the invention the cultured host cell is a prokaryotic cell, such as an *E. coli* cell, a *Lactococcus* cell, a *Lactobacillus* cell or a *Bacillus subtilis* cell, or a eukaryotic cell such as a yeast cell, for example a *Pichia* or *Saccharomyces* yeast cell, or an insect cell, for example after baculoviral infection.

Preferably, an optimised yield of recombinant protein of interest is manifested by increasing the level of *de novo* protein folding.

An optimised yield of said recombinant protein of interest may also be manifested by increasing the level of *in vivo* refolding of aggregated, or misfolded soluble, recombinant protein.

An optimised yield of said recombinant protein of interest may also be manifested by increasing the level of *in vitro* refolding of aggregated, or misfolded soluble, recombinant protein.

An optimised yield of said recombinant protein may also be manifested by increasing the level of *de novo* protein folding in combination with increasing the increased level of *in vivo* refolding and/or *in vitro* protein refolding.

Preferably, said increased level of folding or refolding results in increased solubility of the recombinant protein of interest.

Preferably, said increased level of folding or refolding results in increased activity of the recombinant protein of interest.

According to a third aspect of the present invention there is also provided a method for increasing the degree of refolding of a recombinant protein of interest, said method comprising adding a composition containing a chaperone protein to a preparation of the

recombinant protein of interest *in vitro*. This has been found to increase significantly the degree of refolding of protein in preparations containing wholly or partially unfolded protein. The preparation of the recombinant protein of interest may be any preparation that contains protein that is partially or wholly unfolded or misfolded. Preferably, the preparation is a cell extract preparation, such as a lysate of a prokaryotic cell.

Preferably, a combination of chaperone proteins as described above is added to the preparation of the recombinant protein of interest. For example, such chaperone proteins may include one or more genes encoding one or more proteins selected from the group consisting of the chaperone proteins GroEL, GroES, DnaK, DnaJ, GrpE, ClpB and their homologs (for example, Hsp104, Ydj1 and Ssa1 in yeast), and optionally one or more genes encoding proteins selected from the group consisting of the small heatshock proteins of the IbpA family and/or the IbpB family and/or their homologs.

The preparation of the recombinant protein of interest may be a preparation of soluble recombinant protein that has been precipitated *in vivo*, or may be a preparation of *in vitro* precipitated recombinant protein (for example, a host cell extract containing the recombinant protein aggregate).

Preferably, said composition containing the chaperone protein(s) is added after removal of any agents that are effective to induce soluble recombinant protein expression (such as IPTG for Lac repressor controlled genes) or after addition of a transcription blocking compound (such as glucose for catabolite repressable genes).

Preferably, the third aspect of the invention is used in conjunction with imposing a block in protein synthesis, for example by addition of an effective amount of a protein synthesis inhibitor to the culture system. As described above, chloramphenicol, tetracycline, gentamycin and streptomycin are examples of suitable protein synthesis inhibitors.

Preferably, when practising the above-described methods, the time course of refolding and the temperature at which refolding occurs is controlled. The time course of refolding and temperature at which it occurs are known to have a significant effect on the yield of soluble recombinant protein, and are thus an important aspect of a given expression system to be optimised for the maximal yield of soluble recombinant protein.

Preferably, when practising the above-described methods, a composition containing a protein selected from the group consisting of the small heatshock proteins of the IbpA family and/or the IbpB family and/or their homologs is used in conjunction with the

chaperone proteins GroEL, GroES, DnaK, DnaJ, GrpE, and/or ClpB and/or their homologs.

A further aspect of the present invention relates to methods for the prophylaxis, therapy or treatment of diseases in which aggregated proteins are implicated, comprising the administration of the described combinations of chaperone proteins and/or small heatshock proteins in sufficient amounts. Such diseases include, but are not limited to diseases in which amyloid deposits are implicated, such as late and early onset Alzheimer's disease, SAA amyloidosis, hereditary Icelandic syndrome, multiple myeloma, and spongiform encephalopathies.

Various aspects and embodiments of the present invention will now be described in more detail by way of example. It will be appreciated that modification of detail may be made without departing from the scope of the invention.

Brief description of the Figures

Figure 1A shows chaperone co-overproduction systems tested in *E. coli*. Genes encoding three different chaperone-systems (GroEL/ES; DnaK, DnaJ, GrpE; and ClpB) were cloned in a pair of low copy number vectors, which are compatible with *E. coli* (pSC101 and p15A ori), carry the $lacI^Q$ gene and different resistance markers for selection. Chaperone genes are set under the control of IPTG-regulated promoters (PA11/lacO1) for controlled expression. Each combination of vector pairs (1 to 5) differs in its combination and level of chaperone expression. In these strains subsequently a third plasmid encoding a substrate protein was introduced.

Figure 1B shows chaperone expression patterns. The chaperone combinations 2 to 5 are shown. The left hand lane of each pair is loaded with a sample for which expression of the recombinant proteins had not been induced. The right hand column for each chaperone combination shows an IPTG-induced sample.

Figure 2. Chaperone and target protein co-expression under IPTG control. The target proteins Tep4, Btke and Lzip were purified by metal affinity chromatography after transformation in BL21(DE3) cells used as a control (K) and in the same strain but co-expressing the 5 different chaperone combinations reported in Figure 1.

Figure 3. *In vivo* induced refolding. **Figure 3A** shows the Btke expression level after chaperone-induced re-folding in BL21(DE3) cells used as a control (K) and in the same

strain but co-expressing the 5 different chaperone combinations reported in Figure 1. Cells were grown at 30°C, induced with 0.1 mM IPTG, grown overnight, and then either grown 2 more hours (first lane of each combination) or pelletted, re-suspended in fresh medium plus 200µg/mL chloramphenicol and cultured 2 more hours (second lane). **Figure 3B** shows optimisation of the re-folding conditions using the chaperone combination 4 shown in Figure 3B. After overnight culture at 20°C the cells were pelletted, resuspended in fresh medium and cultured 1h, 2h, 3h, and 4h at 20°C (1 to 4), or 1h and 2h at 37°C (5 and 6) in the presence of 200µg/mL chloramphenicol. For each combination the first lane was loaded with the uninduced sample and the second with the treated one. **Figure 3C** shows Btke expressed in control (C1) and chaperone combination 4 (C2) cells. Lanes were loaded with uninduced samples (K), induced and cultured at 20°C overnight plus two hours at the same temperature , pelleted after overnight growth, resuspended in fresh medium plus 200µg/mL chloramphenicol and cultured 2 more hours, as in 2 but in the presence of 1mM IPTG instead of chloramphenicol, resuspended in fresh medium for 1h, 2h, and 4h. The numbers shown below the gel image indicate the increase factor obtained comparing the intensity of the bands to the reference (induced cells without chaperone co-expression). **Figure 3D** shows the effect of growth conditions on re-folding efficiency of Btke. Cells were grown overnight at 20°C (D1) and at 42°C before inducing the re-folding at 20°C (D2). Lanes were loaded with un-induced samples (K), induced and cultured overnight plus two hours (1), resuspended in fresh medium plus 2h culture (2), in fresh medium plus 200 µg/mL chloramphenicol and cultured 2 more hours (3). **Figure 3E** shows the re-folding efficiency of Tep4 expressed in control (E1) and chaperone combination 4 (E2) cells. Lanes were loaded with uninduced samples (K), induced and cultured overnight plus two hours (1), resuspended in fresh medium plus 2h culture (3), in fresh medium plus 200 µg/mL chloramphenicol and cultured 2 more hours (4).

Fig. 4. *In vitro* re-folding. **Figure 4A** shows Btke expressed either in control cells (c) or in cells co-expressing chaperone combination 3 or 4. 3h after IPTG induction, cells were harvested and lysate prepared as described above. Samples containing 100µg lysate were supplemented with 10mM ATP and 3mM PEP and 20ng/ml PK. After indicated timepoints, soluble Btke protein was isolated and analysed by SDS-PAGE and Coomassie staining. **Figure 4B** shows the results produced when pellets with insoluble Btke were isolated from control cells. Pellets were suspended in buffer and where indicated

chaperones were added. After 5 min, 2, 4, and 20 h soluble Btke protein was isolated as described above and analysed by SDS-PAGE and silver staining.

Figure 5 shows the results of experiments to test the effects of various combinations of different sHSPs and HSPs on the refolding of soluble MDH complexes *in vitro.*

Figure 6 shows the results of experiments to test the effects of different HSP combinations on the refolding of soluble α-glucosidase/sHSP 16.6 and citrate synthase/sHSP 16.6 complexes *in vitro.*

Figure 7 shows the results of experiments to test the effects of different HSP combinations on the refolding of aggregated luciferase and soluble luciferase/sHSP 16.6 complexes *in vitro*

Figure 8 shows the results of KJE/ClpB-mediated refolding of MDH. The different 16.6 concentrations present during MDH denaturation are shown as the indicated 16.6/MDH ratio. Refolding curves for KJE-mediated refolding of MDH are indicated. Refolding curves for refolding of MDH carried out in presence of ClpB/DnaK are differently coloured. The precise 16.6/MDH ratios during MDH denaturation are indicated to the right of the graph and are as follows: green (16.6/MDH ratio=0); light blue (16.6/MDH ratio=0.25); brown (16.6/MDH ratio= 0.5); dark blue (16.6/MDH ratio=1); yellow (16.6/MDH ratio=2); pink (16.6/MDH ratio=4).

Figure 9 shows the results of experiments to determine the effect on protein refolding of varying the concentration of ClpB.

Figure 10 shows the results of experiments to determine the effects of mutations to the ibpAB genes and DnaK genes of *E. coli.*

Figure 11 shows a comparison between the effects of mutations to the ibpAB and clpB genes in *E. coli* on the thermotolerance of those strains.

Figure 12 shows the results of experiments to determine whether IbpA/B protein function increases in importance in the presence of reduced levels of DnaK and at elevated temperatures.

Figure 13 shows the results of experiments to determine the levels of protein aggregation associated with heat shock in Δ*ibpAB* Δ*clpB* double knockout *E. coli* cells.

Figure 14 shows the effect of IpbAB co-expression on the level of soluble target proteins produced in *E. coli* cells.

Figure 15: Effect of plasmid interactions on the level of the recombinant protein expression. A) Recombinant chaperone (K-DnaK, ELS-GroELS, ClpB) accumulation in bacteria homogenates. B) Accumulation of co-expressed recombinant chaperones and target protein GTR1 in the homogenates recovered from control (C) and induced (I) bacteria. C) Effect of chaperone co-transformation on the not induced (C) and IPTG-induced (I) expression of the target protein Btk cloned in pET24d. D) Effect of the co-transformation with an empty pDM1 vector on the not induced expression of Btk cloned in pET24d.

Figure 16: Co-expression of the coil-coiled region of Xklp3A/B. The chains A and B were cloned in a polycistronic vector and expressed either in BL21 (DE3) together with the recombinant chaperone combination K+J+E+ClpB+GroELS (+ chap) or in BL21 (DE3) pLysS in the presence of 1% glucose (-chap).

Figure 17: Effect of unsynchronised recombinant chaperone expression on the level of soluble target recombinant protein. The independent induction of the chaperones and target proteins has been obtained using arabinose-regulated vectors for the target proteins and IPTG-inducible vectors for the chaperones. In the figures are reported the bands corresponding to the soluble target protein purified by affinity chromatography from 0.5 mL of bacterial culture. A) Amount of soluble GTR1 and coiled-coil Xklp3A recovered from wild type cells and bacteria co-transformed with different chaperone combinations. Expression was induced by 0.2 mM IPTG and 1.5 mg/mL arabinose were added 20 min later. The samples were collected 3 hours after the IPTG induction. B) Amount of soluble GTR1 recovered from bacteria co-transformed with K+J+E+ClpB+GroELS and using different combinations of time and expression-inducer concentrations. The samples were collected 3 hours after the addition of the first inducer and the bands corresponding to GroEL are recovered from SDS-gels loaded with the soluble fraction after cell lysis. C) Amount of soluble coiled-coil Xklp3B recovered from bacteria co-transformed with K+J+E+ClpB+GroEL after overnight culture (ON) at 20°C. The replacement of the ON medium with fresh medium (Fr. Md.), 0.2 mM chloramphenicol (Chlor.) and the temperature shift to 30°C were used to stimulate the in vivo re-folding of the aggregated target protein.

Examples

Examples 1-5 below illustrate the materials and methods used to investigate the effect of co-expressing different chaperone combinations on the yield of a large variety of different recombinant proteins.

Example 1: Construction of Chaperone Vectors

Plasmids carrying chaperone genes under the control of the IPTG-sensitive promoter PA1/lacO-1 were constructed as described (Tomoyasu, T., Mogk, A., Langen, H., Goloubinoff, P., Buckau, B., Mol. Microbiol., 40, 397-413, (2001)). Target protein vectors were delivered to the Protein Expression Unit from different research groups working at the European Molecular Biology Laboratory.

Example 2: Transformation procedure

Competent BL21 (DE3) and Top10 cells were transformed with the following couples of plasmids for selective expression of chaperone combinations (Fig.1A). The clones for DnaK, DnaJ, and GrpE were carried by pBB530 and pBB535; the co-expression of DnaK, DnaJ, GrpE, and ClpB was regulated by pBB535 and pBB540; GroEL/ES system was expressed by pBB528 and pBB541; a large amount of the complete system DnaK, DnaJ, GrpE, ClpB, and GroEL/ES was ensured by pBB540 and pBB542; finally, a lower expression level of the same chaperone combination was obtained using pBB540 and pBB550. A complete array of single chaperone plasmid transformed cells was also prepared as a control. Transformed cells were checked for chaperone expression and successively made competent. The protease deficient strain BB7333 (MC4100 Δ*clpX,* Δ*clpP,* Δ*lon*) was used for transforming the Btkp protein. These strains were also made competent and used for a further transformation with the target proteins.

Example 3: Cell cultures

Single colonies from the transformed cells were used to inject 3 mL of LB medium. Liquid cultures were performed initially at 37°C, then transferred to 30°C and finally transferred to 20°C. Using different times of incubation at the higher temperatures it was possible to reach the OD_{600} of 0.8 at the same time for all the different cell strains cultured together for comparative expression assays. Protein expression was performed overnight by inducing gene transcription using 0.1 mM IPTG. 1.5 mL of the overnight culture of both IPTG-

induced (hereafter termed 'induced) and control bacteria was directly centrifuged in an Eppendorf tube and the pellet frozen and stored at –20°C. Alternatively, the pellet was re-suspended in 3 mL of fresh medium and divided into two aliquots of 1.5 mL, with or without the addition of 200 μg/mL chloramphenicol. After 2h culture at 20°C the cells were harvested as described before. Inclusion body overproduction was obtained by culturing the bacteria at 42°C overnight after induction. Large scale cultures were grown in 2L flasks using 5 mL of overnight LB pre-culture to inoculate 500 mL of Terrific Broth.

Example 4: Protein purification and evaluation

Frozen bacterial pellets were re-suspended in 350 μL of 20 mM Tris HCl, pH 8.0, 2mM PMSF, 0.05% Triton X-100, 1μg/mL DNAase and 1 mg/mL lysozyme and incubated on ice for 30 min, with periodic stirring. The suspension was sonicated in water for 5 minutes, an aliquot (of homogenate) was stored and the rest was pelleted in a minifuge. An aliquot of the supernatant was preserved and the rest was added to 15 μL of pre-washed magnetic beads (Qiagen) and incubated further 30 min under agitation before being removed. Beads were washed 30 min with 20 mM K-phosphate buffer, pH 7.8, 300 mM NaCl, 20 mM imidazole, 8% glycerol, 0.2%Triton X-100 and later with PBS buffer plus 0.05% Triton X-100. Finally they were boiled in 12 μL SDS sample buffer and the samples loaded for SDS PAGE analysis, using a Pharmacia minigel system. Proteins were detected after coloration with Simply Blue Safestain (Invitrogen) following the manufacturer's instructions and the gels were recorded using a Umax Astra 4000U scanner. Bands corresponding to the proteins were analysed using the public NIH Image 1.62f software. Alternatively, protein was eluted from washed beads using 30μL PBS buffer plus 0.5M imidazole and its relative concentration measured following its adsorbance at 280nm. The proper folding was evaluated by circular dichroism using a J-710 spectropolarimeter (Jasco).

Example 5: In vitro experiments

Cells were grown in LB and after inducing the synthesis of either Btke or Btke together with GroEL/ES (combination 3) or together with GroEL/ES, DnaK, DnaJ, GrpE, ClpB (combination 4) for 3h with 1mM IPTG at 37°C, lysates were prepared as described above. For refolding of Btke from inclusion bodies using total lysate, 10mM ATP, 3mM phosphoenole pyruvate (PEP) and 20 ng/ml pyruvate kinase (PK) were added and incubated at 20°C. After 5 min, 2, 4, and 20 h soluble material was separated from

insoluble fractions by centrifugation (15 min, 4°C, 10.0000rpm) and the soluble fraction was used to isolate target protein as described above.

For resolubilsation of isolated Btke aggregates with exogenous chaperone addition, 100µg of total lysate (isolated from cells with overproduced Btke) was centrifuged for 15 min and pellets were resuspended in 20 mM Tris/HCl, 100mM KCl and 20 mM MgCl. Chaperones were added as indicated and samples incubated at 20°C for 5 min, 2, 4, 20h. Soluble material was separated from inclusion bodies by centrifugation and isolated as described.

Examples 6 to 9 below illustrate the optimisation of chaperone co-expression combinations and other experimental variables in order to greatly increase the yield of a large number of diverse recombinant proteins.

Example 6: Investigation of the Effect of Chaperone Combinations on *de novo* Protein Folding

Five different combinations of plasmids encoding chaperone systems (GroEL/ES; DnaK, DnaJ, GrpE and ClpB) in different combinations and amounts under the control of IPTG regulated promoters were introduced into BL21 (DE3) cells as illustrated in Fig. 1A. The degree of chaperone expression was shown to be very high (Fig. 1B). These cells were subsequently transformed with plasmids expressing substrate proteins in an IPTG controlled manner (Fig. 1A). Therefore, co-expression of chaperones and target proteins was obtained by simultaneous induction of all the promoters with IPTG. Co-expression of chaperones together with 50 different target genes was tested. For each target protein, all five different chaperone combinations were tested and solubility of the recombinant proteins analysed. In summary a higher yield of soluble substrate protein was achieved in more than 50% of the tested constructs (see Table 1 below).

Table 1 shows a list of the proteins used in the survey for analysing the effect of chaperone co-expression on soluble target protein yield. The table shows the molecular weight of the constructs, the original organisms from which they were cloned, whether they corresponded to full length proteins (Fl) or to domains, expressed alone or fused to a partner (fus), and their cell localisation (cytoplasm, membrane, nucleus, secreted) *in vivo*. The yield increase factor (IF) induced by the best chaperone combination is reported under 'Chap. IF' and the yield increase factor obtained using the refolding protocol under 'Refolding IF'. The symbol (/) signifies that the experiment has not yet been done and (!)

that protein has been obtained using constructs that gave no soluble protein when expressed in wild type bacteria.

Table 1:

Protein	MW	Organism	Features	Chap. IF	Refolding IF
GTR1	40 kD	S. cerevisiae	Fl/cyt	3	3
BtKp	55 kD	H. sapiens	domain/cyt	0	28
Xpot1	110 kD	H. sapiens	Fl/cyt	0	/
XklpA1	62 kD	X. laevis	domain/fus/cyt	0	!
XklpB1	40 kD	X. laevis	domain/fus/cyt	0	!
HbpH	9 kD	H. sapiens	domain/cyt	3.5	3.5
TEVprotease	30 kD	TEV	domain	3.5	/
Pex5p	50 kD	H. sapiens	domain/cyt	0	/
UCP1	33 kD	R. norvegicus	domain/membr	0	0
Transcr Fact	37 kD	H. sapiens	Fl/cyt	0	0
BtKe	55 kD	H. sapiens	domain/cyt	4	42
XklpA2	38 kD	X. laevis	domain/fus/cyt	0	/
XklpB2	35 kD	X. laevis	domain/fus/cyt	0	/
XklpA3	72 kD	X. laevis	domain/fus/cyt	0	/
Rolled	43 kD	D. melanogaster	Fl/cyt	4.5	4.5
Lzip	41 kD	H. sapiens	Fl/cyt	!	/
1Ap	52 kD	D. melanogaster	Fl/nucl	0	/
Chip	64 kD	D. melanogaster	Fl/nucl	0	/
dLMO	37 kD	D. melanogaster	Fl/nucl	0	/
Tlc	57 kD	R. prowazekii	Fl/membr	0	/
BtKc	64 kD	H. sapiens	Fl/cyt	3	/
PhosphK	29 kD	H. sapiens	Fl/cyt	3	7
Compl.Tep3	47 kD	A. gambiae	domain/fus	4	/
Compl.Tep4	45 kD	A. gambiae	domain/fus	3.5	/
XklpA4	72 kD	X. laevis	domain/fus/cyt	2.5	2.5

XklpB3	73 kD	X. laevis	domain/fus/cyt	2.5	2.5
E8R1	58 kD	Vaccinia virus	Fl/membr/fus	7	/
Compl.Tep3	70 kD	A. gambiae	domain/fus/secr	0	11
Compl.Tep4	68 kD	A. gambiae	domain/fus/secr	3.5	13
MaxF	7.5 kD	syntetic	domain	3	/
XklpA5	35 kD	X. laevis	domain/fus/cyt	0	19
E8R2	85 kD	Vaccinia virus	Fl/membr/fus	5.5	5.5
Susy	90 kD	Z. mays	Fl/membrane	3	5
Mash	91 kD	Z. mays	Fl/cyt	0	3
PPAT	22 kD	E. coli	Fl/cyt	0	3
2Ap	54 kD	D. melanogaster	Fl/nucl	!	3
F10L	45 kD	Vaccinia virus	Fl/fus	0	0
B1R	47 kD	Vaccinia virus	Fl/fus	3.5	3.5
1Frenge	43 kD	D. melanogaster	domain/cyt	!	!
Tep1	7 kD	A. gambiae	domain/secr	3	6
Tep2	11 kD	A. gambiae	domain/secr	0	0
2Frenge	55 kD	D. melanogaster	domain/fus	0	2
GFP-fusion	95 kD	A. victoria	Fl/fus/cyt	0	0
2C18	50kD	H. sapiens	Fl/fus	3	8
22j21	72kD	H. sapiens	Fl/fus	!	!
XklpA+B	15+17kD	X. laevis	domain/complex	2.5	3.5
Msl3	14 kD	D. melanogaster	domain/cyt	2.5	2.5
Mash+Susy	94+90 kD	Z. mays	Fl/complex	3	3
Endostatin	22 kD	M. musculus	domain/secr	0	0
Kringle	30 kD	H. sapiens	domain/fus	0	0

As can be seen from the 'Chap. IF' ratings, soluble target protein yield increased between 2.5 and 7-fold. Effects of co-expressed chaperones were not limited to a certain type of substrate protein. The target proteins tested were representative of several different classes, including complexes, soluble, membrane-bound and secreted proteins, full-length, domains

and fusion constructs, with a molecular weight spanning from 7.5 to 110 kD, expressed in the cytoplasm and in the periplasm (Table 1). Moreover, in some cases, like Lzip (see Table 1 and also Figure 2), co-expression of chaperones was the only possibility to obtain any soluble protein. Evaluation of the 23 positive cases indicated that the most efficient chaperone combination was the fourth, which expressed all three chaperone systems in large amounts, followed by the third, fifth, first and the second. Nevertheless, as is demonstrated in the case of LZip transcription factor where chaperone combination 1 worked far better than the others, any one chaperone combination is not necessarily optimal for all target proteins. Thus, despite the systematic approach it was not possible to infer general rules about the optimal conditions to succeed. No protein class showed better results in combination with particular chaperone combinations and no expression vector ensured significantly better yields. The only exception was when target proteins were cloned in high copy number vectors. In such a case no positive result was observed. The competition for the protein synthesis machinery could be considered as a reason, since chaperone expression is inhibited when a target protein was co-expressed and is completely prevented in cells harbouring expression vectors with pUC origin (data not shown). The results shown in Table 1 clearly demonstrate the very large increases in yield possible via the use of the disclosed methods.

Example 7: Testing the Effect of Co-overexpression of Chaperone Combinations and Target Proteins on Re-folding of Aggregated Proteins Using Chloramphenicol

In the experiments of Example 5 it was often observed that inclusion bodies accumulated even in the presence of overproduced chaperones increasing the amount of soluble proteins. A recent paper (Carrio, M. M. and Villaverde, A. FEBS Lett., 489, 29-33 (2001)) showed that soluble proteins could be recovered *in vivo* from inclusion bodies when the protein synthesis was blocked by chloramphenicol addition and the whole cellular folding machinery became available for precipitated proteins. Therefore, we investigated the overexpression of chaperones not only for keeping recombinant proteins soluble but also for increasing the re-folding capability of cells. To investigate this further, we co-overexpressed chaperones and target genes as described before. Subsequently, we stopped protein synthesis by the addition of chloramphenicol. Cells were transferred to fresh media, incubated at 20°C and resolubilisation of targets had been analysed at different time points. In fact, in the case of Btke the chloramphenicol-induced block of protein synthesis induced

a low increment of the soluble recombinant protein in control cells but an impressive increase when specific chaperone combinations were co-expressed simultaneously with the target gene prior to the translational arrest (Fig. 3A). It is worthy to note that for Btke the optimal chaperone combination differed when the soluble protein accumulated during standard culture conditions and when protein synthesis has been blocked (Figs. 2 and 3A). The choice of time and temperature conditions during re-folding was crucial for optimising the result (Fig. 3B). Longer incubation times or higher temperature lowered the amount of recovered soluble protein, probably because degradation by proteases takes over re-folding activity. As can been seen in Table 1 above, this method of combining chaperone co-overexpression with the blocking of protein synthesis resulted in a great improvement in the yield of recombinant protein in a large number of the combinations tested.

Example 8: Testing the Effect of Co-overexpression of Chaperone Combinations and Target Proteins on Re-folding of Aggregated Proteins by Reducing Construct Gene Transcription

The protocol used to block protein synthesis, as described in Example 6 above, was evaluated by means of experiment. It was found that the original protocol can be simplified and that it was not strictly necessary to completely prevent protein synthesis in order to induce re-folding, and in fact the cessation of recombinant protein expression by removing the induction agent (IPTG) was sufficient. In this case the target protein could be re-folded to a level comparable to that obtained in the presence of chloramphenicol but only in the presence of the recombinant over-expressed chaperones (Fig. 3C). For Btke the optimal re-folding conditions enabled the recovery of 42-fold more protein than in the standard growth conditions using normal BL21 (DE3) cells and the simplified protocol (without chloramphenicol) gave an increase factor of 26. We also tried to induce the inclusion body formation culturing the bacteria at 42°C and starting the re-folding from a higher amount of material but the improvement was negligible (Fig. 3D), probably indicating that the limiting factor is represented from the folding machinery or from the cellular degrading metabolism. These two factors seem to be somehow connected, as illustrated in the case of Tep4. In contrast to Btke this protein was expressed in soluble form at sufficient levels also at standard culture conditions and chaperone co-expression induced a limited yield increase (Fig. 3E). Nevertheless, the suppression of IPTG induction by simple exchange with fresh medium boosted the accumulation of soluble protein in both the strains but only the co-expression of recombinant chaperones could ensure the same results when

chloramphenicol was added. Generally, we observed that the addition of fresh medium alone was more effective than the combination of fresh medium and chloramphenicol in strains with wild type chaperone expression. This indicates that the removal of the inducer IPTG, and the subsequent cessation of transcription of the target gene, is sufficient to allow refolding from inclusion bodies. It was a goal of the inventors to obtain more information about the relationship between protein re-folding and degradation by transforming our vectors in the protease deficient strain BB7333. However, the inventors were not able to raise a sufficient number of bacterial colonies. This finding confirmed the general role of proteases in maintaining cell viability (Tomoyasu, T., Mogk, A., Langen, H., Goloubinoff, P., Buckau, B., Mol. Microbiol., 40, 397-413, (2001)) and suggests that a certain degree of protein degradation must be maintained. It is therefore clear from the above example that a reduction in recombinant target gene transcription can also allow the refolding of aggregated proteins to proceed, leading to greatly improved yields of the soluble recombinant protein of interest.

Protein synthesis inhibitors other than chloramphenicol, such as tetracycline, gentamycin and streptomycin have been tested with similar effects.

Example 9: The Effect of Co-overexpression of Chaperone Combinations and Target Proteins on Re-folding of Aggregated Proteins *in vitro*

Next, we analysed whether co-expressed chaperones are capable of enhancing the refolding of target proteins from inclusion bodies *in vitro* after cell lysis. For that purpose, we induced simultaneously synthesis of Btke together with either chaperone combination 3 or 4. Cells were harvested after induction and total lysates containing inclusion bodies and chaperones were isolated. Subsequently an ATP-regenerating system was added to the lysates and the soluble protein was purified after 5 min, 2h, 4h and 20h. Lysate containing the chaperone mixture 4, which was the most efficient during the *in vivo* refolding of Btke, showed already 5 min after the addition of ATP that approximately all Btke could be recovered in the soluble fraction. The control lysate, where only Btke was overexpressed, and the lysate with enhanced levels of GroEL/EL showed no significant recovery of soluble Btke (Fig. 4A). It is therefore clear that co-overexpressed chaperone mixtures stimulate re-solubilisation of inclusion bodies from bacterial cell lysates. Refolding of Btke inclusion bodies was also possible when chaperones were added exogenously to isolated aggregates (Figure 4B). However, refolding efficiency was much lower and refolding

kinetics much slower, most probably due to the limited amount of added chaperones. This example clearly shows that co-expression of chaperones can also increase the yield of soluble recombinant protein via an enhancement of the refolding of target proteins from aggregates/inclusion bodies *in vitro*.

The above Examples 1-8 have clearly shown the value of the methods provided by the present invention for increasing protein yield. The re-folding protocol applied to the chaperone transformed cells allowed even higher yields of soluble protein than the simple co-expression with the target proteins in 8 on 17 cases and, importantly, also gave positive results also in the case of 8 constructs insensitive to simple co-expression. Taking all the results together chaperones had a positive effect on soluble protein accumulation in 68% of the cases analysed in our survey. The ratio remains basically the same if all the 50 constructs are considered (34 positive) or if only the 37 different proteins are taken in account (24 positive, 65%). It must be remarked that such a positive result has been obtained despite the fact that most of the constructs used in the experiment correspond to sequences difficult to be expressed in a soluble form in bacteria, like membrane-associated or secreted proteins, regions not corresponding to structural domains or complexes (underlined in Table 1). The advantage of the *in vivo* disaggregation is that protein refolding follows native patterns and, therefore, recovers its native conformation. The correct folding of some of the proteins was analysed by purification until homogeneity followed by circular dichroism analysis, indicating that the proteins had adopted their native conformation after refolding. Importantly, the enzymatic activities of the kinases B1R and F10L, the TEV protease and luciferase were also recovered after re-folding (data not shown). Larger scale cultures confirmed the trend observed in test cultures, suggesting that the disclosed methods are suitable for industrial applications. In summary, the invention provides not only a method for the production of large amounts of soluble recombinant protein, but also a method for the production of large amounts of recombinant protein that is correctly folded and furthermore retains the native protein's biological activity.

In the following examples 10 and 11, the effect of small heat shock proteins (sHSPs) on the yield of soluble recombinant proteins both *in vitro* and *in vivo* was investigated. Published data had previously shown that members of the chaperone family of small heat

shock proteins (sHSPs), such as the *E. coli* family members IbpA and IbpB (IbpAB), can efficiently prevent the aggregation of unfolded proteins, although they were not shown to exhibit protein refolding activity. In the present study, refolding of substrates from sHSP/substrate complexes is reported to be dependent on an Hsp70 chaperone system (such as DnaK with its DnaJ and GrpE co-chaperones) in a reaction that can be further stimulated by the GroEL and GroES (GroELS) chaperones.

Example 10: Investigation of the effect of small heat shock proteins on the yield of soluble recombinant proteins *in vitro*

The refolding of several recombinant proteins from soluble complexes was tested:

Materials and Methods:

1 μM MDH was denatured in buffer A (50 mM Tris pH 7.5; 150 mM KCl; 20 mM $MgCl_2$) for 30 min at 47°C either in the presence of 6 μM 18.1 (pea), or 6 μM IbpB (*E. coli*), or 4 μM 16.6 (*Synechocystis* sp.). MDH refolding was initiated at 30°C by adding an ATP regenerating system (2 mM ATP; 3 mM PEP; 20 ng/ml pyruvate kinase) and various chaperone combinations made up from KJE (1 μM DnaK; 0.2 μM DnaJ; 0.1 μM GrpE), ESL (4 μM GroEL; 4 μM GroES) and ClpB (1.5 μM). The results for these experiments are shown in Figure 5.

Similarly, 1 μM α-glucosidase or 1 μM citrate synthase were denatured in the presence of 4 μM 16.6 (*Synechocystis* sp.) in buffer A for 45 min at 50°C or 47°C, respectively. Protein refolding was initiated at 30°C by adding an ATP regenerating system (2 mM ATP; 3 mM PEP; 20 ng/ml pyruvate kinase) and various chaperone combinations made up from KJE, ESL and ClpB. The results for these experiments are shown in Figure 6.

Similarly, 100 nM firefly luciferase was denatured in the absence or presence of 0,4 μM 16.6 (*Synechocystis* sp.) in buffer A for 15 min at 43°C. Luciferase refolding was initiated at 30°C by adding an ATP regenerating system (2 mM ATP; 3 mM PEP; 20 ng/ml pyruvate kinase) and various chaperone combinations made up from KJE (0.5 μM DnaK; 0.1 μM DnaJ; 0.05 μM GrpE) and ClpB (0.5 μM). The results for these experiments are shown in Figure 7.

To investigate the effect of the stoichiometry of the sHSPs on the refolding of sHSP/substrate complexes 1 μM MDH was denatured in buffer A (50 mM Tris pH 7,5; 150 mM KCl; 20 mM $MgCl_2$) for 30 min at 47°C in the presence of varying 16.6

concentrations. MDH refolding was initiated at 30°C by adding an ATP regenerating system (2 mM ATP; 3 mM PEP; 20 ng/ml pyruvate kinase) and various chaperone combinations made up from KJE (1 μM DnaK; 0.2 μM DnaJ; 0.1 μM GrpE) and ClpB (1.5 μM). The results for these experiments are shown in Figure 8.

Experiments were also carried out in which 1 μM MDH was denatured in buffer A (50 mM Tris pH 7.5; 150 mM KCl; 20 mM $MgCl_2$) for 30 min at 47°C in the absence or presence of 0.5 μM 16.6. MDH refolding was initiated at 30°C by adding an ATP regenerating system (2 mM ATP; 3 mM PEP; 20 ng/ml pyruvate kinase) and the DnaK system (1 μM DnaK; 0,2 μM DnaJ; 0,1 μM GrpE) in the presence of varying ClpB concentrations as indicated. The results for these experiments are shown in Figure 9.

Results:

All the sHSPs tested formed complexes with heat-denatured protein substrates such as malate dehydrogenase (MDH), firefly luciferase and alpha-glucosidase which represented small protein aggregates. The data shown in Figure 5 show that ClpB strongly stimulates the DnaK-dependent refolding of the thermolabile reporter protein malate dehydrogenase (MDH) from various soluble sHSP/MDH complexes. This stimulatory effect was verified by analysis of the refolding of the substrates firefly luciferase, citrate synthase and α-glucosidase from complexes with sHSP 16.6 (shown in Figure 6 and Figure 7). Notably, the refolding of substrates by ClpB/DnaK from sHSP/substrate complexes was in general much faster than refolding from aggregated proteins generated by identical denaturation conditions in the absence of sHSPs (Figure 7). The GroESL chaperone system was not able to refold any of the substrates tested from sHSP/substrate complexes, even in the presence of ClpB. However GroESL was observed to increase the rates of substrate refolding in the presence of DnaK or ClpB/DnaK, especially in case of MDH (Figure 5). Table 2 provides a summary of the results from these experiments:

Table 2: Refolding of thermolabile proteins from protein aggregates or soluble sHsp/protein complexes

Substrate	Chaperones			
	KJE	KJE/ESL	KJE/ClpB	KJE/ClpB/ESL
aggr. MDH	0.1	0.2	10.3	25.1
sHsp/MDH	4.0	9.9	8.5	27.5
aggr. α-glucosidase	0	0	1.73	2.27
sHsp/α-glucosidase	0.44	0.53	2.69	3.63
aggr. citrate synthase	0	0	0.06	0.1
sHsp/citrate synthase	0.12	0.22	0.4	0.63
aggr. luciferase	0.01	n.d.	0.14	n.d.
sHsp/luciferase	0.17	n.d.	0.48	n.d.
	Refolding rate (nM/min)			

MDH, α-glucosidase, citrate synthase and luciferase were denatured in the absence or presence of a 4-fold excess of 16.6. Substrate refolding was initiated by addition of an ATP-regenerating system and the indicated chaperone combinations (experimental details as described above). Maximal rates of substrate refolding were derived from the linear phase of the time curves of recovered enzymatic activity.

On the basis of these results, we propose that sHSP/substrate complexes represent small protein aggregates and refolding of substrates from such complexes relies on a disaggregation reaction mediated by the DnaK system alone, or much more efficiently by ClpB with the DnaK system. After their active extraction from the complex, unfolded

substrates are subsequently refolded by a chaperone network formed by the DnaK and GroESL systems.

In vivo the levels of sHSPs are often not sufficient to prevent protein aggregation and sHSPs are usually found associated with protein aggregates. We investigated whether the presence of sHSPs in protein aggregates can facilitate their resolubilization and consequently increase substrate refolding. To answer this question the amount of sHSPs utilised in each experiment was titrated during the denaturation of MDH and the resulting consequences on DnaK or DnaK/ClpB-mediated MDH refolding were investigated. Substoichiometric concentrations of Hsp16.6 compared to MDH resulted in the formation of insoluble, turbid sHSP/MDH complexes which were, however, much smaller than MDH aggregates formed by denaturation in the absence of Hsp16.6 (Table 3).

Table 3: Characterisation of 16.6/MDH complexes

			Size determination	
			Dynamic lightscattering	**Static lightscattering**
16.6/MDH Ratio	**Lightscattering intensity (%)**	**Solubility (%)**	**Calculated radius (nm)**	**Mass (Da)**
0	100	<10	45 +/- 15	n.d.
0.25	68	<10	33.7 +/- 12.5	1.8E+07 - 7.0E07
0.5	37	18	31.5 +/- 9	1.8E+07 - 7.0E+07
1	0	57	24 +/- 6	5.6E+07 - 1.5E+07
2	0	84	19 +/- 5	2.3E+06 - 4.0E+06
4	0	92	14 +/- 5	1.5E+06 - 3.1E+06

1 µM MDH was denatured in buffer A (50 mM Tris pH 7,5; 150 mM KCl; 20 mM $MgCl_2$) for 30 min at 47°C in the presence of varying 16.6 concentrations, given as 16.6/MDH ratio. Turbidity (light scattering intensity) of formed MDH aggregates was set at 100%. Solubility of native, untreated MDH after centrifugation (13.000 rpm, 15 min, 4°C) was set 100%. Size of the different sHSP/substrate complexes were determined either by dynamic or static lightscattering (coupled to gelfiltation) measurements. Both techniques were utilised in case of poorly soluble sHSP/MDH complexes leading to characterization of a subpopulation of the complexes only.

Increasing Hsp16.6 concentrations increased the solubility and decreased turbidity and size of sHSP/MDH complexes (Table 3). Efficient DnaK-dependent MDH refolding required the presence of soluble sHSP/MDH complexes created in the presence of high Hsp16.6 concentrations (Figure 8). In contrast ClpB/DnaK mediated MDH refolding did not show up such a severe dependency, however MDH activity was recovered at earlier timepoints if insoluble sHSP/MDH complexes instead of MDH aggregates were used as starting material. This effect became much more severe, if the disaggregation potential of the ClpB/DnaK system was reduced by lowering the ClpB concentration (Figure 9). The stimulatory effects described above were again observed when substoichiometric concentrations of sHSPs were present during substrate denaturation (by heat), resulting in the formation of insoluble sHSP/substrate complexes. Thus the presence of sHSPs in insoluble protein aggregates can significantly facilitate aggregate resolublization by ClpB/DnaK.

The above example illustrates that refolding of substrates after their ClpB/DnaK mediated extraction from sHSP/substrate complexes is in most cases stimulated by the GroESL chaperone system, indicating that released, unfolded substrates are refolded by a chaperone network. We conclude that sHSP function is coupled to ClpB/DnaK dependent protein disaggregation and serves to prepare protein aggregates for faster resolubilization.

Example 11: Investigation of the effect of small heat shock proteins on the yield of soluble recombinant proteins *in vivo*

Materials & Methods:

E. coli wild type or Δ*ibpAB* or Δ*dnaK* mutant cells were grown at 30°C to logarithmic phase and shifted to 45°C for 30 min, followed by a recovery phase at 30°C for 60 min. Protein aggregates were isolated at the indicated timepoints and analyzed by SDS-PAGE. The results for these experiments are shown in Figure 10.

E. coli wild type or Δ*ibpAB* or Δ*clpB* or Δ*ibpAB* Δ*clpB* double mutant strains were grown at 30°C to logarithmic phase. Cells were either shifted directly to 50°C or were preincubated at 42°C for 15 min. Various dilutions of stressed cells were plated on LB plates. After 18 h colony numbers were counted and survival rates were calculated in

relation to determined cell numbers before 50°C shock. The results for these experiments are shown in Figure 11.

. Various dilutions (10^{-3} to 10^{-6}) of the cultures were spotted on LB plates supplemented with the indicated IPTG concentrations and incubated at 30°C, 37°C or 42°C for 18 h. The results for these experiments are shown in Figure 12.

Various strains of *E. coli* were grown overnight at 30°C in the presence of 500 µM IPTG. Cultures were washed twice with LB and inoculated for further growth at 30°C in the presence of various IPTG-concentrations (0, 25, 50, 100 µM) to logarithmic phase and shifted to 42°C for 30 min. Protein aggregates were isolated at the indicated timepoints and analyzed by SDS-PAGE. The results for these experiments are shown in Figure 13.

In the experiments described above in examples 6-9 we expressed in *E. coli* strain BL21(DE3) several target proteins including 2C18, E8R, Tep3 and Kringle with or without co-expression of different combinations of the chaperones GroELS, ClpB, DnaK, DnaJ and GrpE. The chaperone combination which for each case yielded the highest levels of soluble target proteins was taken as "control" (overproduction of KJE/ELS/B for 2C18, Tep3, no chaperone overproduction for E8R and Kringle). To show the solubilization effects of overproduction of IbpA/IbpB together with other chaperones we generated BL21(DE3) strains which carry plasmids expressing IPTG-regulatable genes encoding these same target proteins and in addition plasmids expressing IPTG regulatable genes encoding IbpA/IbpB (lanes marked IbpAB in Figure 14), IbpA/IbpB and GroELS (lanes marked IbpAB+GroELS in Figure 14), IbpA/IbpB and GroELS and DnaK/DnaJ/GrpE and ClpB (lanes marked IbpAB+compl. in Figure 14). After IPTG induction the bacteria were cultured overnight at 20°C and directly collected (I), or the IPTG was removed and the pellet re-suspended in fresh medium and cultured for two additional hours without (N) or with 200 µg/ml of chloramphenicol (C). For each combination the amount of soluble protein (after affinity purification of the target proteins in the soluble cell fractions) was identified on Coomassie-stained SDS-gels. The results for these experiments are shown in Figure 14.

Results:

E. coli mutant cells missing the sHSPs IbpA/B do not exhibit a temperature-dependent growth phenotpye (42°C). However, we observed that the resolubilization of protein aggregates, created by severe heat treatment (45°C), was delayed in comparison to wild

type cells (Figure 10). Additionally the survival rate (thermotolerance) of Δ*ibpAB* mutants at lethal temperatures (50°C) was slightly reduced compared to wild type (Figure 11). Thermotolerance is linked to the ability of cells to rescue aggregated proteins and consequently the observed reduced thermotolerance of Δ*ibpAB* mutants is likely caused by a less efficient resolubilization of protein aggregates.

DnaK has been shown to be the major player in preventing protein aggregation in *E. coli* at high temperatures. We therefore investigated whether IbpA/B function could become more important in the presence of reduced DnaK levels, rendering *E. coli* cells more sensitive to protein aggregation. *In vivo* depletion of DnaK was achieved by replacing the σ32-dependent promotor of the *dnaKJ* operon by an IPTG-inducible one. Reduced DnaK levels caused synthetic lethality in Δ*ibpAB* mutant cells at elevated temperatures (37-42°C). The same experiments performed in a Δ*clpB* mutant strain and a Δ*ibpAB* Δ*clpB* double knockout revealed an increasing necessity for higher DnaK levels at elevated temperatures (Figure 12). Especially in case of the Δ*ibpAB* Δ*clpB* double knockout mutant strain this phenotype was linked to severe protein aggregation upon heat shock to 42°C (Figure 13). Thus *in vivo* IbpA/B is necessary for efficient protein disaggregation, especially under conditions which favour protein aggregation and lower the disaggregation potential of cells.

As shown in Figure 14 and Table 4, the combined overproduction of IbpAB with ClpB, the DnaK system and the GroEL system, and with combinations of these chaperones, increases the yield of soluble recombinant protein produced in *E. coli* cells.

Table 4:

Protein	MW	Organism	Features	IpbAB IF
SerprotAg1		A. gambiae	domain/fus	!
Kringle	30 kD	H. sapiens	domain/fus	!
2C18	50kD	H. sapiens	Fl/fus	**2.5**
22j21	72kD	H. sapiens	Fl/fus	**0**
Tep3	70 kD	A. gambiae	domain/fus/secr	**3.5**
Tep4	68 kD	A. gambiae	domain/fus/secr	**0**
XklpA3	73 kD	X. laevis	domain/fus/cyt	**0**
E8R1	58 kD	Vaccinia virus	Fl/membr/fus	**3.5**
BtKe	55 kD	H. sapiens	domain/cyt	**0**

Nine proteins were tested for the effects of IpbAB co-expression on the level of soluble target proteins produced in *E. coli* cells. The increment factor (IF) defines the fold increase (in the best condition, being either I, N or C; see above for definition) in amount of soluble protein due to IpbAB co-expression with respect to the controls (the best conditions identified from examples 6-9). ! denotes that the IpbAB-dependent expression of soluble proteins occurred which could not be produced in soluble form before. Thus, in 5 of the nine cases tested, the overproduction of IbpA/IbpB further increasesd the yield of target proteins.

Thus, these *in vivo* data are consistent with the results obtained *in vitro*. Firstly, the yields of soluble recombinant protein produced in *E. coli* cells can be increased in several cases tested when IbpA/IbpB is overproduced alone or together with various combinations of the DnaK and GroELS systems and ClpB. Secondly, *E. coli* Δ*ibpAB* mutant cells missing IbpA/B exhibited a delayed protein disaggregation after heat shock (45°C) and a reduced survival rate at lethal temperatures (50°C) compared to wild type cells. IbpA/B function

became essential at elevated temperatures (37-42°C) in the presence of reduced DnaK levels, conditions which favour protein aggregation and reduce the disaggregation potential of the cells.

In summary, the above Examples 10 and 11 show that small heat shock proteins (sHSPs) co-operate with other chaperones, in particular with the ClpB chaperone, the DnaK chaperone system and the GroEL chaperone system, to solubilize and refold aggregation-prone proteins. This property can be exploited to increase the yield of soluble recombinant proteins produced in *E. coli* and other cells, and can be used for the *in vitro* production of soluble recombinant protein. In particular, the combined overproduction of IbpAB with ClpB, the DnaK system and the GroEL system, and with combinations of these chaperones, increases the yield of soluble recombinant protein produced in *E. coli* cells. However, the teaching provided by these experiments is of much broader importance since all the proteins involved in this folding reaction are members of large protein families with members among prokaryotes and eukaryotes (IbpA and IbpB are members of the family of sHSPs which includes alpha-cristallins; ClpB is member of the AAA protein family which include Hsp104; DnaK is member of the Hsp70 family; DnaJ is member of the DnaJ (Hsp40) family; GrpE is member of the GrpE family; GroEL is member of the Hsp60 family; GroES is member of the GroES family). It is expected that the other members of the involved protein families can substitute for the *E. coli* members in protein folding reactions. In fact, we present biochemical data that the sHSP of *Synechocystis*, Hsp16.6, can increase the efficiency of protein refolding in co-operation with the *E. coli* chaperones ClpB, DnaK, DnaJ, GrpE, and GroELS. Furthermore, since ClpB is a homolog of the *S. cerevisiae* Hsp104, a chaperone implicated in the generation and prevention of formation of amyloid fiber formation, it is also possible that our finding that the sHSPs co-operate with ClpB and the DnaK and GroEL systems in protein folding has implications on the formation or treatment of amyloid fibers in eukaryotic cells, and diseases in which such fibers are implicated.

Finally, it was found that the IbpA/B, ClpB and the DnaK systems act cooperatively to reverse protein aggregation.

To elucidate the functional interplay between IbpA/B, KJE and ClpB in the protein quality control network more precisely, we determined the degree of protein aggregation in Δ*ibpAB*, Δ*clpB* and Δ*ibpAB* Δ*clpB* mutants that have KJ adjusted to various levels. Since

ClpB and IbpA/B do not prevent protein aggregation *in vivo* (Mogk et al., 1999), increased amounts of aggregated proteins in the respective mutants would indicate a less efficient protein disaggregation. At 30°C no protein aggregation was detectable for all tested mutant strains, even in cells with greatly reduced KJ levels. After a 30 min incubation at 42°C, 5% of cellular proteins aggregated in all mutant cells, provided that IPTG was omitted from the growth medium. Increasing KJ levels (by addition of IPTG) reduced the amount of aggregated proteins in each strain, but to different degrees dependent on the mutant background. While 50 μM IPTG in the growth medium was sufficient to eliminate aggregates in Δ*ibpAB* cells, 2% and 5% of total proteins still aggregated in Δ*clpB* and Δ*ibpAB* Δ*clpB* mutant cells, respectively. Even in presence of DnaK/DnaJ levels corresponding to heat shock conditions (100 μM IPTG), 2% of cellular proteins remained aggregated in Δ*ibpAB* Δ*clpB* mutant cells.

These findings are in complete agreement with the hierarchial complementation of growth defects of these mutant cells at high temperatures and demonstrate the cooperative action of IbpA/B and ClpB in the KJE-mediated removal of protein aggregates *in vivo*.

Example 12: Bacteria co-transformed with recombinant proteins and chaperones cloned in independent plasmids are suitable for expression tuning

This example describes a system based on three vectors, where two are under IPTG regulation and enable the recombinant expression of six chaperones, and the third one is arabinose-inducible and harbours the sequence for the recombinant target protein of interest. In such a way, the independent induction and the level of expression of both chaperones and target protein was possible. The data showed that the expression leakage from pET vectors was prevented by the introduction of further plasmids in the cell and that the recombinant proteins compete for their expression. In fact, the high rate induction of one of them could switch off the accumulation of the other recombinant proteins. The first information was used to maximise the expression of toxic proteins while the cross-inhibition among recombinant proteins was exploited to modulate and optimise the target protein expression and to induce the chaperone-assisted in vivo re-folding of aggregated target protein.

Cloning and transformation procedures.

Chaperone proteins were expressed as described above. For expression of target protein, the sequences corresponding to GTR1 (O 00582) and the motor regions of Xklp3A and Xklp3B (AJ 311602; CAA 08879) were cloned in pTrcHis vector (trc promoter and ColE1 replication origin), Tep3 (unpublished sequence from A. gambiae) was cloned in pGEX (tac promoter and pBR322 replication origin) and E8R (NP 063710) in pGAT (lac promoter and pUC replication origin). The sequences for GTR1, Tep3, E8R, the Xklp3A and B C-terminal regions of the coil-coiled domains and a domain of Btk (O 06187) were cloned in pBAD. pET24d and pETM60. The Xklp3A and B C-terminal regions of the coil-coiled domains were also cloned in the polycistronic vector pST39 (Tan, 2001).

Cell cultures.

Single colonies from the transformed cells were used to inject 3 mL of LB medium. Liquid cultures were incubated initially at 37°C, successively transferred to 30°C or 20°C, induced at an OD_{600} of 0.8 and grown 3 hours or overnight, respectively. Variations of timing and concentration combinations used in the experiments with bacteria hosting both IPTG and arabinose regulated expression vectors are described case by case in the results.

Protein purification and yield evaluation.

Frozen bacterial pellets corresponding to 0.5 mL of culture were re-suspended in 350 µL of 20 mM Tris HCl, pH 8.0, 2mM PMSF, 0.05% Triton X-100 and 1 mg/mL lisozyme and incubated on ice for 30 min, with periodic stirring. The suspension was sonicated in water for 5 minutes, pelleted in a minifuge, the supernatant was added to 20 µL of pre-washed Ni-NTA magnetic agarose beads (Qiagen) and incubated further 30 min under agitation before being removed. Beads were washed 30 min with 20 mM K-phosphate buffer, pH 7.8, 300 mM NaCl, 20 mM imidazole, 8% glycerol, 0.2%Triton X-100 and later with PBS buffer plus 0.05% Triton X-100. Finally they were boiled in 12 µL SDS sample buffer and the samples loaded onto a SDS PAGE using a Pharmacia minigel system. Proteins were detected after coloration with Simply Blue Safestain (Invitrogen) following the manufacturer's instructions and the gels were recorded using a Umax Astra 4000U scanner. Proteins were quantified analysing the gel bands with the public domain NIH Image program (developed at the U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/).

Results and Discussion

Initially we transformed bacteria with chaperone-carrying plasmids and in a second step these cells were re-transformed with a plasmid harbouring the target protein. The expression of all the plasmids was under IPTG regulation. The cell co-transformation with three plasmids selected using different antibiotic resistances induced a 20% decrease of the cell growth rate; however, succeeded even in the case in which two plasmids (pGAT and pBAD) shared the same replication origin pUC.

Bacteria transformed with two low copy number plasmids derived from pDM1 and harbouring different chaperone genes expressed the corresponding proteins at very high level (Fig. 15A). Nevertheless, the intensity of the bands separated in SDS-gel indicated that the expression of the target protein GTR1 cloned into the pTrcHis vector strongly inhibited the chaperone accumulation (compare Fig. 15A and 15B) so that ClpB was no more detectable in the bacterial homogenate (Fig. 15B). In contrast, the expression of the target protein Btk by the leaking vector pET24d in the absence of the inducer IPTG was strongly repressed when a chaperone-containing plasmid was co-transformed in the host cell (Fig. 15C). These results suggest that there are two independent kinds of interaction raising from the presence of different plasmids in the same cell. The first one involves the plasmids and is independent from their protein products. In fact, the IPTG-independent expression of Btk cloned in a pET24d expression vector was prevented also in the case of the co-transformation with an empty pDM1 vector (Fig 15D). A recent paper reports that the introduction of heterologous vectors has been shown to induce stress responses and inhibit biomass production in S. cerevisiae even though they were empty or non-induced (Görgens *et al.*, 2001, Biotechnol. Bioeng. 73, 238-245).

The expression-leakage control obtained by co-transformation with more plasmids at once can be useful in the case of the expression of toxic proteins or when the leakage rate is so high to impair the normal cell function. At least one experience in the frame of this work indirectly supports this hypothesis. A polycistronic plasmid (Tan, 2001) has been used for expressing a complex between the C-terminal end of the coil-coiled regions of Xklp3 chain A and chain B. No colony grew using BL21 (DE3) bacteria when we tried to transform them with the polycistronic plasmid. Cells co-transformed with chaperone plasmids were efficiently transformed with the polycistronic vector and gave colonies. Colonies grew also when the polycistronic vector was transformed into pLysS strain cells and 1% glucose was added to the growth medium to tightly control any expression leakage. However, the

bacterial yield was 60% less (data not shown) and the purified protein decreased of more than 80% (Fig. 16).

Beside the case of plasmid interaction our results seem to indicate that the cell machinery involved in the protein production was challenged by the contemporary over-expression of too many recombinant proteins. The results of Figure 15A and 15B could be interpreted either as an overwhelming accumulation of target protein transcripts that inhibits the chaperone expression rate or a competition for the RNA polymerase. Such a competition has been described in *E. coli* between metabolic and recombinant genes (Schweder *et al.*, 2002, Appl. Microbiol. Biotechnol. 58, 330-337) while recombinant and cell mRNAs could compete at transcriptional level in yeast (Görgens *et al.*, 2001, Biotechnol. Bioeng. 73, 238-245). We observed that in case of co-transformation the effect of competition seems proportional to the estimated copy number of the target protein plasmid and independent on the promoter used (data not shown). In fact, recombinant vectors hosting the target protein with both T7 and lac promoters could inhibit the chaperone expression. Therefore, a competition at the transcriptional level would be ruled out in our system. The existence of a limit of total protein expression can have important consequences in the case in which recombinant chaperones are co-transformed to boast the production of a target protein. In fact, a too high level of expression of the latter could automatically inhibit the chaperone expression levels and, therefore, limit or prevent their positive folding effect.

An alternative method has been envisaged in which chaperones and target proteins were cloned in vectors in which their expression was under different regulation systems. This enables the independent induction of chaperone and target protein expression and would allow exploitation of the chaperone-dependent folding improvement of the target protein avoiding any shortcomings due to contemporary co-expression. A logical approach seemed to induce the accumulation of the chaperones and then trigger the target protein expression in a cell with boasted folding machinery.

In a first set of experiments the GroELS chaperones were expressed by means of an arabinose-regulated vector (Castanié *et al.*, 1997, Anal. Biochem. 254, 150-152) and the IPTG-dependent target proteins were induced after 30 minutes. The results did not show a significant increase of soluble target proteins and no improvement was detected varying incubation times and inducer concentrations (data not shown).

In a second attempt the target proteins were cloned into arabinose-regulated vectors while five different IPTG-dependent chaperone combinations were compared. Such an expression system mostly resulted in an increased yield of the soluble target proteins (see Table 5).

Protein	MW	Organism	Improvement Factor
GTR1	40 kD	S. cerevisiae	3
Btkp	55 kD	H. sapiens	3
Xklp3A	62 kD	X. laevis	∞
Xklp3B	40 kD	X. laevis	9
Tep3	70 kD	A. gambiae	4
E8R	32 kD	Vaccinia virus	0

Table 5. Chaperone-dependent yield improvement of soluble target proteins. Clones corresponding to the target proteins were co-transformed with the different chaperone combinations and cultured according to the best among the conditions reported in Figure 17. The improvement factor enabled by chaperone co-expression indicates the ratio between the highest yield of soluble target protein obtained using cells co-transformed with chaperones and its amount recovered from cells not hosting recombinant chaperones; the symbol ∞ means that no soluble target protein was expressed in absence of chaperones. The target proteins were expressed in arabinose-regulated pBAD vectors and the different chaperone combinations listed in material and methods were induced by IPTG addition.

The optimal chaperone combination (Fig. 17A) and the expression conditions were specific for each target protein. The complexity of the interactions among the different recombinant proteins is illustrated in the experiments summarised in Figure 17B and 17C. Soluble GTR1 accumulation was induced at a similar level by both 0.5 and 1.5 mg/mL of arabinose (Fig. 17B, lanes 1 and 2). The co-expression of low amounts of K+J+E+ClpB+GroELS chaperones induced by 0.02 mM IPTG stimulated the accumulation of soluble GTR1 whose expression was induced by 0.5 mg/mL of arabinose (Fig. 17B, lane 4).

Nevertheless, the amount of the soluble target protein decreased if IPTG-dependent chaperones were allowed to accumulate before the arabinose-dependent induction of GTR1 (Fig. 17B, compare lanes 4 and 5). The same pattern of inhibition of soluble GTR1 was observed when higher chaperone expression was induced by ten-fold higher IPTG concentration but, in such a case, the absolute amount of soluble GTR1 was strongly reduced (lanes 6 and 7). These data confirm the existence of a competition among the products of different recombinant plasmids. In this case, both plasmids use the cell RNA polymerase and, therefore, it is not possible to distinguish between competition at the transcription or translation level. Nevertheless, the accumulation of soluble GTR1 induced at low level of arabinose is progressively inhibited by an increasing amount of available chaperones (Fig. 17B). The inhibitory chaperone accumulation was obtained with both higher IPTG concentration and longer time of induction before the arabinose-dependent induction of GTR1. When we repeated the same experiments using 1.5 mg/mL of arabinose to induce a higher GTR1 expression the results were reversed (Fig. 17B, compare lanes 8-11 and 4-7). As a matter of fact the higher arabinose concentration enabled a strong accumulation of GTR1; however, the increasing amounts of expressed chaperones did not reach a level critical for competition but could provide a more stabilising environment for GTR1.

The conclusions from this work are that chaperones can positively contribute to GTR1 accumulation. Nevertheless, a ratio among the transcripts seems to be important for avoiding detrimental competition at the translation level. The parameters involved are the rate of induction of both chaperone and target genes and the time in which chaperones can accumulate before the target protein is induced.

Recently, it has been showed that recombinant proteins precipitated in aggregates could be re-solubilised *in vivo*. Aggregate re-folding was induced after that translation inhibition made available foldases and chaperones otherwise employed in metabolic folding (Carrìò and Villaverde, 2001, FEBS Letts. 489, 29-33). We applied this idea to our system in which the accumulation of recombinant chaperones was possible.

The expression of coiled-coil Xklp3B was induced overnight at 0.5 mg/mL of arabinose (Fig 17C, lane 1). The amount of recovered soluble protein was low and inhibited or almost completely prevented when chaperones (K+J+E+GroELS+ClpB) expression was IPTG-induced together or before arabinose addition (Fig. 17C, lanes 2 and 3). These data

confirm the results collected using GTR1 and explained considering a competition among the recombinant proteins (Fig. 17B). In contrast, the removal of the arabinose-containing medium and the addition of fresh medium plus chloramphenicol had a positive effect on the amount of recovered soluble protein (Fig. 17C, lane 4). Apparently, the standard cellular folding machinery is, therefore, sufficient to partially re-fold the aggregated recombinant target protein. Nevertheless, a strong re-solubilisation improvement of the target protein was observed only when arabinose was removed, the pellet was re-suspended in fresh medium and chaperone-expression was induced by 0.2 mM IPTG addition (Fig. 17C, lane 5). A similar improvement at a slightly lower extent was obtained by the simple addition of a sufficiently high amount of IPTG (0.2 mM) to the arabinose-containing medium (Fig. 17C, lane 6). Therefore, it seems that it is possible to exploit the inhibitory effect of an overwhelming chaperone expression on the arabinose-regulated target protein to switch the system from Xklp3B to chaperone expression. Then, in conditions that inhibit the further expression of Xklp3B (comments to Fig. 17B), the available chaperones induce the re-folding of the already aggregated target protein without the need to remove the arabinose from the medium.

The collected results provide new information concerning the co-transformation of more than one recombinant proteins and confirm that chaperone co-transformation can increase the amount of soluble target protein. They also indicate that interactions among transformed plasmids and among corresponding proteins need to find an equilibrium in the host cell to optimise the co-transformation benefit. In fact, it seems that chaperones can somehow compete with the target protein, meaning that some care is required to optimise each candidate system, although this is well within the ambit of the skilled worker. Nevertheless, the reciprocal expression inhibition between target protein and chaperones can be exploited to tune the expression rate and improve the amount of soluble target protein. We must only be aware that the conditions need to be optimised since the accumulation rate is specific for each recombinant protein.

CLAIMS

1. A method for the expression of a recombinant protein of interest, said method comprising:

    a) culturing a host cell which expresses:

        i) one or more genes encoding the recombinant protein(s) of interest;

        ii) at least two genes encoding proteins selected from the group consisting of the chaperone proteins GroEL, GroES, DnaK, DnaJ, GrpE, ClpB and their homologs (for example, Hsp104, Ydj1 and Ssa1 in yeast); under conditions suitable for protein expression; and

    b) separating said recombinant protein of interest from the host cell culture.

2. A method according to claim 1, wherein the genes selected in step a) ii) include DnaK, DnaJ and GrpE or homologs thereof.

3. A method according to claim 2, wherein the genes selected in step a) ii) additionally include ClpB or a homolog thereof.

4. A method according to any one of claims 1-3, wherein the genes selected in step a) ii) include GroES and GroEL or homologs thereof.

5. A method according to claim 4, wherein the genes selected in step a) ii) include the DnaK, DnaJ, GrpE, ClpB, GroES and GroEL genes or homologs thereof.

6. A method for the expression of a recombinant protein of interest, said method comprising:

    a) culturing under conditions suitable for protein expression a host cell which expresses:

        i) one or more genes encoding one or more recombinant protein(s) of interest;

        ii) one or more genes encoding proteins selected from the group consisting of the chaperone proteins GroEL, GroES, DnaK, DnaJ, GrpE, ClpB and their homologs (for example, Hsp104, Ydj1 and Ssa1 in yeast);

iii) one or more genes encoding proteins selected from the group consisting of the small heatshock proteins of the IbpA family and/or the IbpB family and/or their homologs; and

b) separating said recombinant protein of interest from the host cell culture.

7. A method according to any one of the preceding claims wherein the levels of the respective chaperone proteins are controlled.

8. A method according to claim 7, wherein said levels of chaperone proteins are controlled by expressing the genes encoding the respective chaperone proteins from different promoters.

9. A method according to claim 7 or claim 8, wherein the respective chaperone proteins are expressed using expression systems of different strength.

10. A method according to any one of claims 7-9, wherein said chaperone proteins are over-expressed relative to the expression levels that occur naturally in non-recombinant cells.

11. A method according to any one of the preceding claims, wherein the levels of the chaperone proteins relative to the recombinant protein(s) of interest are controlled by expressing the genes encoding the respective proteins from different promoters or by using different polymerases.

12. A method according to any one of the preceding claims, wherein in culturing step a) of the method, a block in protein synthesis is imposed, for example, by the addition of an effective amount of a protein synthesis inhibitor to the culture system, once a desired level of recombinant protein of interest has accumulated.

13. A method according to claim 12, wherein the chosen protein synthesis inhibitor is chloramphenicol, tetracycline, gentamycin or streptomycin.

14. A method according to any one of claims 1-13, wherein in culturing step a) of the method, a reduction in gene transcription is imposed, for example, by removal of any agents that are effective to induce recombinant protein expression (such as IPTG for Lac repressor controlled genes), or via the addition of a transcription blocking compound (such as glucose for catabolite repressable genes), once a desired level of recombinant protein of interest has accumulated

15. A method for the expression of a recombinant protein of interest, said method comprising:

a) culturing a host cell which expresses:

i) one or more genes encoding the recombinant protein(s) of interest;

ii) one or more genes encoding one or more proteins selected from the group consisting of the chaperone proteins GroEL, GroES, DnaK, DnaJ, GrpE, ClpB and their homologs (for example, Hsp104, Ydj1 and Ssa1 in yeast); under conditions suitable for protein expression;

b) imposing a block in protein synthesis, for example, by the addition of an effective amount of a protein synthesis inhibitor to the culture system, once a desired level of recombinant protein of interest has accumulated; and

c) separating said recombinant protein of interest from the host cell culture.

16. A method for the expression of a recombinant protein of interest, said method comprising:

a) culturing a host cell which expresses:

i) one or more genes encoding the recombinant protein(s) of interest;

ii) one or more genes encoding one or more proteins selected from the group consisting of the chaperone proteins GroEL, GroES, DnaK, DnaJ, GrpE, ClpB and their homologs (for example, Hsp104, Ydj1 and Ssa1 in yeast); under conditions suitable for protein expression;

b) imposing a reduction in gene transcription, for example, by removal of any agents that are effective to induce recombinant protein expression (such as IPTG for Lac repressor controlled genes), or via the addition of a transcription blocking compound (such as glucose for catabolite repressable genes), once a desired level of recombinant protein of interest has accumulated; and

c) separating said recombinant protein of interest from the host cell culture.

17. A method according to claim 15 or claim 16, wherein said host cells additionally expresses one or more genes encoding proteins selected from the group consisting of the small heatshock proteins of the IbpA family and/or the IbpB family and/or their homologs.

18. A method according to any one of claims 14 to 17, wherein in step a) ii), a combination of chaperone proteins is expressed as recited in any one of claims 2-6.

19. A method according to any one of claims 15, claim 17 or claim 18, wherein the chosen protein synthesis inhibitor is chloramphenicol, tetracycline, gentamycin or streptomycin.

20. A method according to any one of the preceding claims, wherein said cultured host cell is a prokaryotic cell, such as an *E. coli* cell, a *Lactococcus* cell, a *Lactobacillus* cell or a *Bacillus subtilis* cell, or a eukaryotic cell such as a yeast cell, for example a *Pichia* or *Saccharomyces* yeast cell, or an insect cell, for example after baculoviral infection.

21. A method according to any one of the preceding claims, wherein an optimised yield of said recombinant protein of interest is manifested by increasing the level of *de novo* protein folding.

22. A method according to any one of claims 1-20, wherein an optimised yield of said recombinant protein of interest is manifested by increasing the level of *in vivo* refolding of aggregated, or misfolded soluble, recombinant protein.

23. A method according to any one of claims 1-20, wherein an optimised yield of said recombinant protein of interest is manifested by increasing the level of *in vitro* refolding of aggregated, or misfolded soluble, recombinant protein.

24. A method according to claim 20, wherein an optimised yield of said recombinant protein is manifested by increasing the level of *de novo* protein folding in combination with an increased level of *in vivo* protein refolding and/or *in vitro* protein refolding.

25. A method according to any one of claims 21-24, wherein said increased level of folding or re-folding results in increased solubility of the recombinant protein of interest.

26. A method according to any one of claims 21-25, wherein said increased level of folding or re-folding results in increased activity of the recombinant protein of interest.

27. A method for increasing the degree of refolding of a recombinant protein of interest, said method comprising adding a composition containing a chaperone protein to a preparation of the recombinant protein of interest *in vitro*.

28. A method according to claim 27, wherein a combination of chaperone proteins as recited in any one of claims 2-6 is added to the preparation of the recombinant protein of interest.

29. A method according to claim 27 or claim 28, wherein the preparation of the recombinant protein of interest is a preparation of soluble recombinant protein that has been precipitated *in vivo*.

30. A method according to claim 27 or claim 28, wherein the preparation of the soluble recombinant protein of interest is a preparation of *in vitro* precipitated recombinant protein.

31. A method according to any one of claims 27-30, wherein said composition containing the chaperone protein(s) is added after removal of any agents that are effective to induce soluble recombinant protein expression (such as IPTG for Lac repressor controlled genes) or after addition of a transcription blocking compound (such as glucose for catabolite repressable genes).

32. A method according to any one of claims 27-31, additionally comprising the step of imposing a block in protein synthesis, such as by the addition of an effective amount of a protein synthesis inhibitor to the culture system.

33. A method according to claim 32, wherein the chosen protein synthesis inhibitor is chloramphenicol, tetracycline, gentamycin or streptomycin.

34. A method according to any one of the preceding claims, wherein the refolding temperature and time course of refolding are controlled.

35. A method according to any one of claims 27-34, additionally comprising the use of one or more proteins selected from the group consisting of the small heatshock proteins of the IbpA family and/or the IbpB family and/or their homologs

36. The use of one or more genes encoding one or more proteins selected from the group consisting of the chaperone proteins GroEL, GroES, DnaK, DnaJ, GrpE, ClpB and their homologs (for example, Hsp104, Ydj1 and Ssa1 in yeast), and one or more genes encoding proteins selected from the group consisting of the small heatshock proteins of

the IbpA family and/or the IbpB family and/or their homologs, in the manufacture of a medicament for the treatment of disease in which the presence of aggregated proteins are implicated.

37. The use of one or more selected from the group consisting of the chaperone proteins GroEL, GroES, DnaK, DnaJ, GrpE, ClpB and their homologs (for example, Hsp104, Ydj1 and Ssa1 in yeast), and one or more genes encoding proteins selected from the group consisting of the small heatshock proteins of the IbpA family and/or the IbpB family and/or their homologs, in the manufacture of a medicament for the treatment of disease in which the presence of aggregated proteins are implicated.

38. A method of treating a patient suffering from a disease in which the presence of aggregated proteins is implicated, comprising administering one or more genes encoding one or more proteins selected from the group consisting of the chaperone proteins GroEL, GroES, DnaK, DnaJ, GrpE, ClpB and their homologs (for example, Hsp104, Ydj1 and Ssa1 in yeast), and one or more genes encoding proteins selected from the group consisting of the small heatshock proteins of the IbpA family and/or the IbpB family and/or their homologs.

39. A method of treating a patient suffering from a disease in which the presence of aggregated proteins is implicated, comprising administering one or more proteins selected from the group consisting of the chaperone proteins GroEL, GroES, DnaK, DnaJ, GrpE, ClpB and their homologs (for example, Hsp104, Ydj1 and Ssa1 in yeast), and one or more proteins selected from the group consisting of the small heatshock proteins of the IbpA family and/or the IbpB family and/or their homologs.

40. The method of claim 38 or claim 39, wherein the disease is late or early onset Alzheimer's disease, SAA amyloidosis, hereditary Icelandic syndrome, multiple myeloma, or a spongiform encephalopathy.

FIG. 1(A)

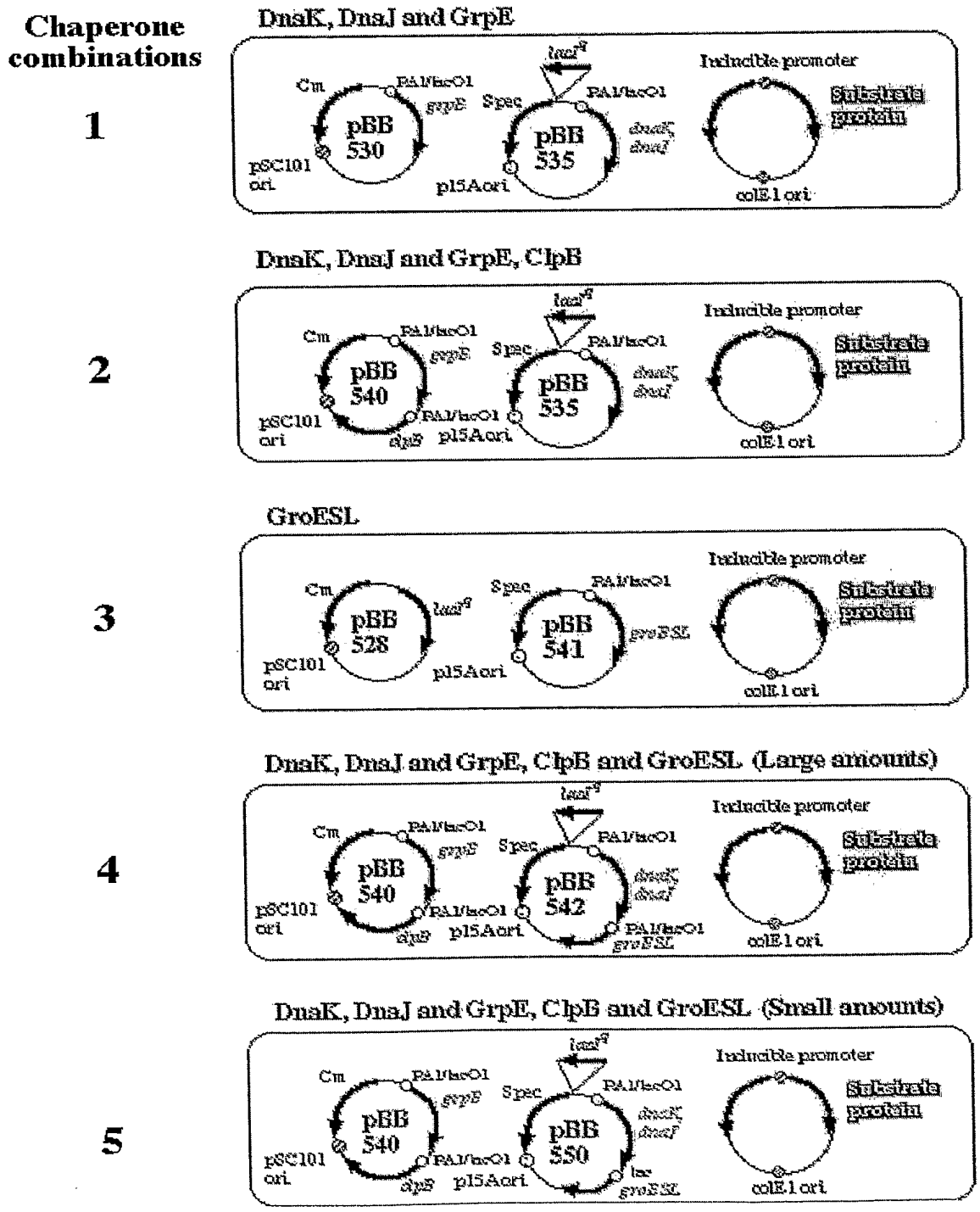

Chaperone combinations
1
DnaK, DnaJ and GrpE
Cm
PA1/lacO1
grpE
Spec
lacI^q
PA1/lacO1
dnaK
dnaJ
pBB 530
pBB 535
pSC101 ori
p15Aori
Inducible promoter
Substrate protein
colE1 ori
2
DnaK, DnaJ and GrpE, ClpB
Cm
PA1/lacO1
grpE
Spec
lacI^q
PA1/lacO1
dnaK
dnaJ
pBB 540
pBB 535
pSC101 ori
PA1/lacO1
clpB
p15Aori
Inducible promoter
Substrate protein
colE1 ori
3
GroESL
Cm
lacI^q
pBB 528
pSC101 ori
p15Aori
Spec
PA1/lacO1
groESL
pBB 541
Inducible promoter
Substrate protein
colE1 ori
4
DnaK, DnaJ and GrpE, ClpB and GroESL (Large amounts)
Cm
PA1/lacO1
grpE
Spec
lacI^q
PA1/lacO1
dnaK
dnaJ
pBB 540
pBB 542
pSC101 ori
PA1/lacO1
clpB
p15Aori
PA1/lacO1
groESL
Inducible promoter
Substrate protein
colE1 ori
5
DnaK, DnaJ and GrpE, ClpB and GroESL (Small amounts)
Cm
PA1/lacO1
grpE
Spec
lacI^q
PA1/lacO1
dnaK
dnaJ
pBB 540
pBB 550
pSC101 ori
PA1/lacO1
clpB
p15Aori
lac
groESL
Inducible promoter
Substrate protein
colE1 ori

FIG. 1(B)

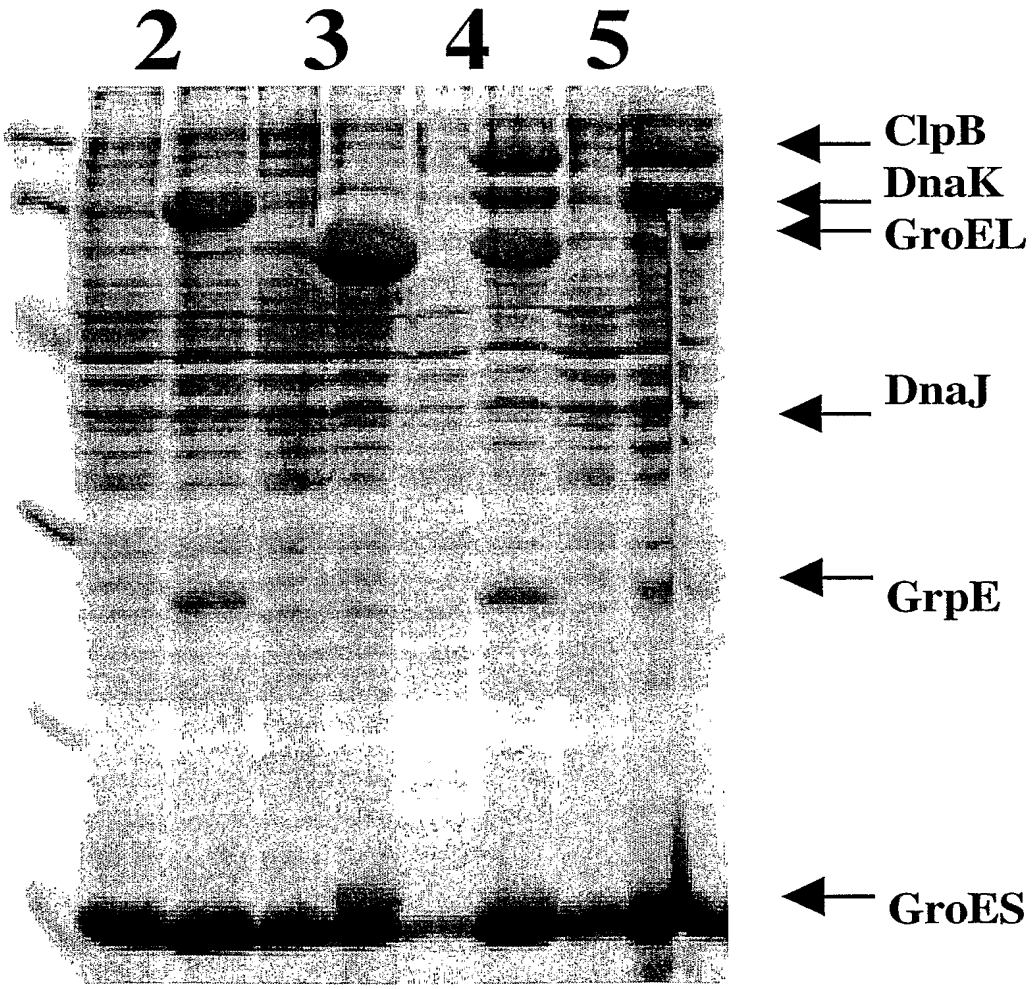


FIG. 2

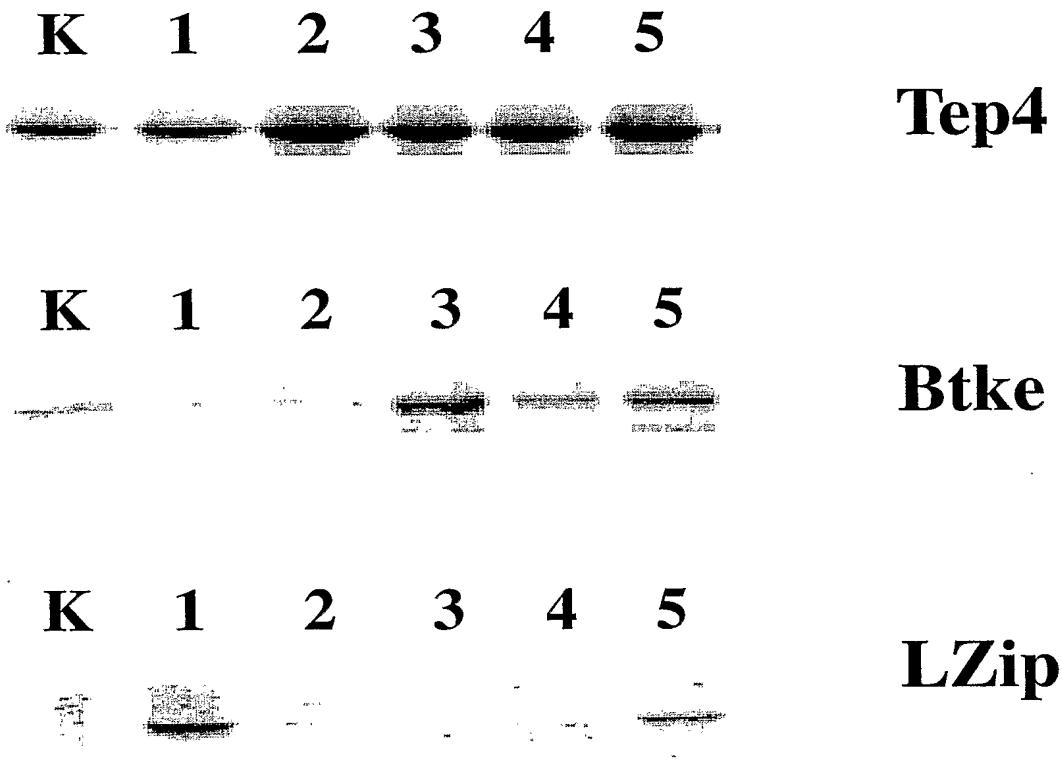
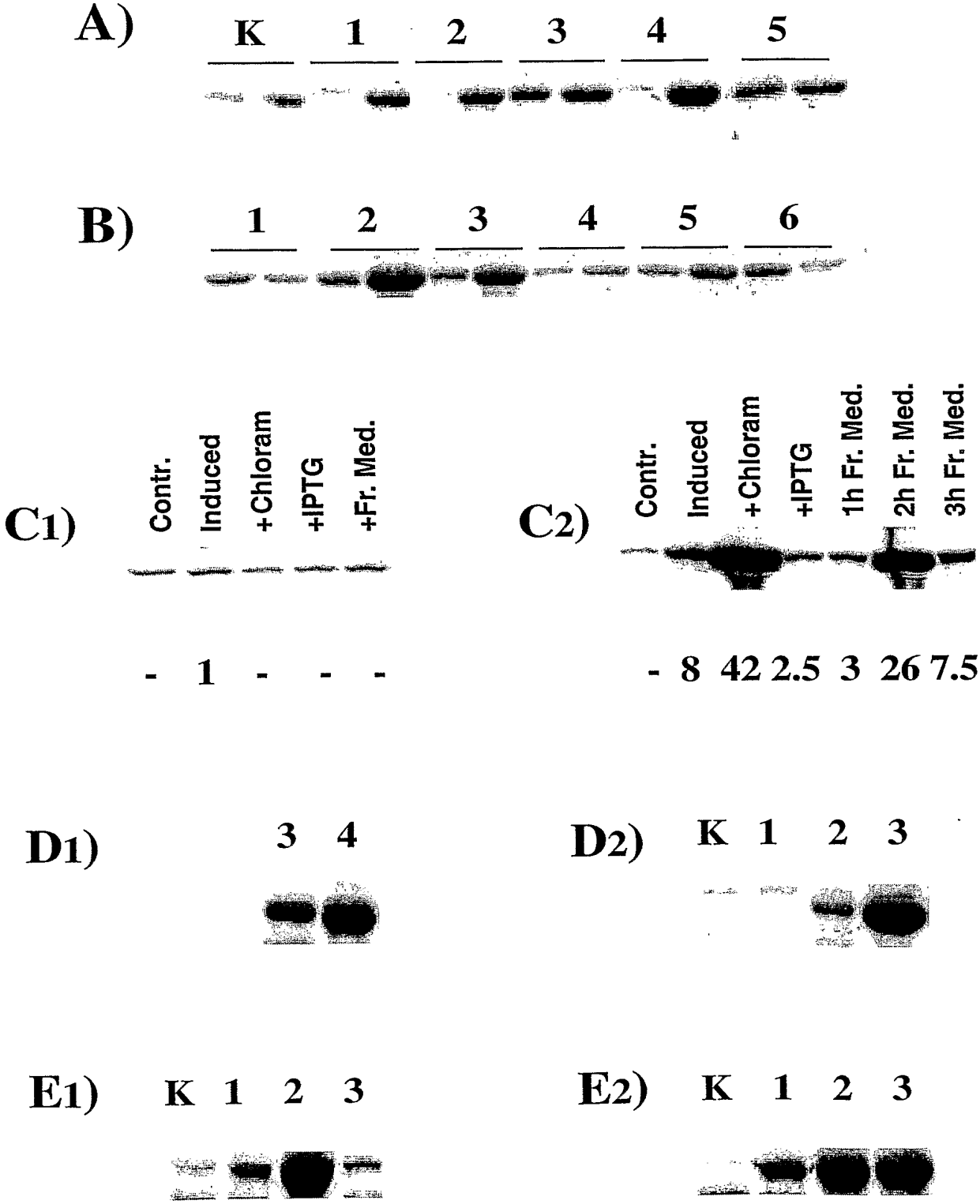


FIG. 3


A)
K 1 2 3 4 5
B)
1 2 3 4 5 6
C1)
Contr.
Induced
+Chloram
+IPTG
+Fr. Med.
- 1 - - -
C2)
Contr.
Induced
+Chloram
+IPTG
1h Fr. Med.
2h Fr. Med.
3h Fr. Med.
- 8 42 2.5 3 26 7.5
D1)
3 4
D2)
K 1 2 3
E1)
K 1 2 3
E2)
K 1 2 3

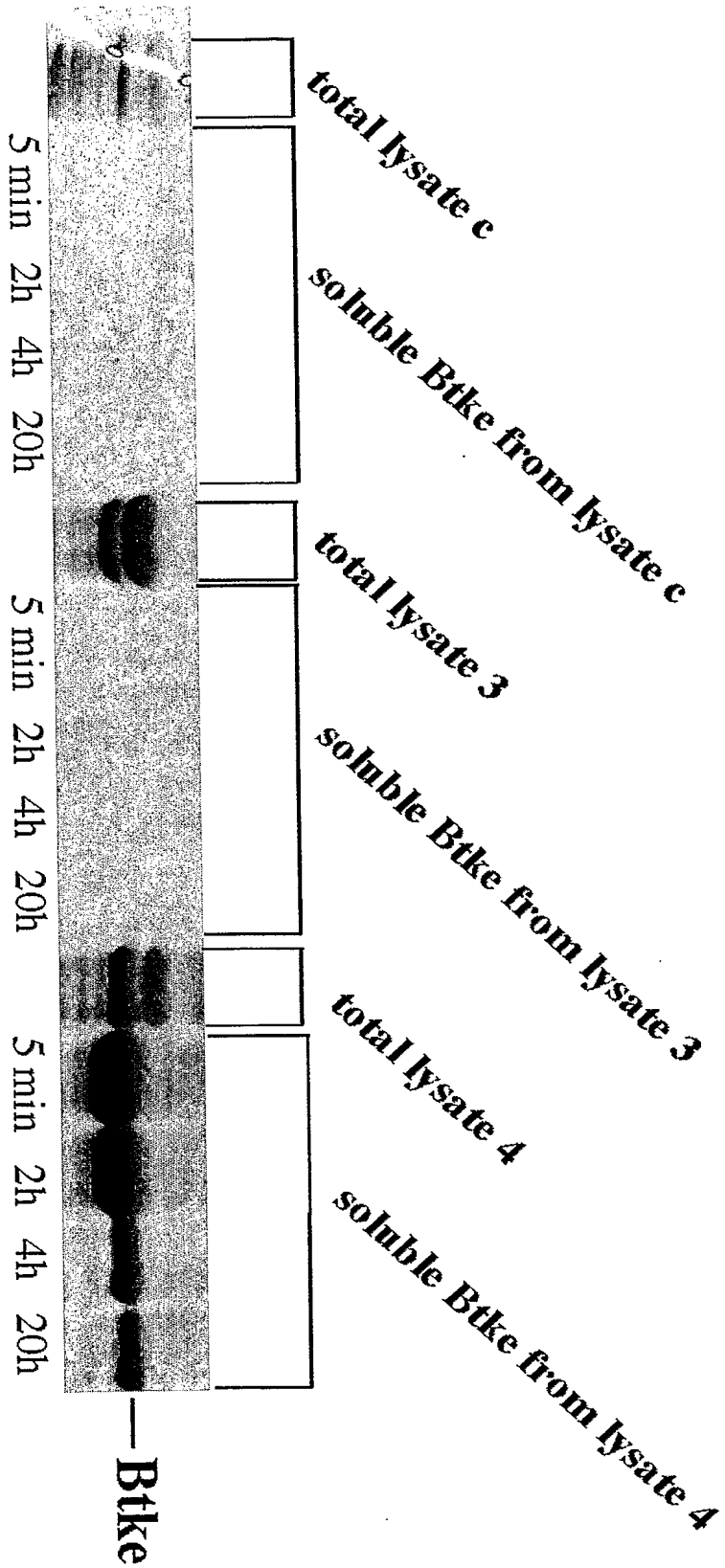

total lysate c
soluble Btke from lysate c
total lysate 3
soluble Btke from lysate 3
total lysate 4
soluble Btke from lysate 4
5 min
2h
4h
20h
5 min
2h
4h
20h
5 min
2h
4h
20h
Btke

FIG. 4(A)

FIG. 4(B)

Incubation time	5 min	2h	4h	20h
regnerating system (10mM ATP, PK, PEP)	+	+	+	+
DnaK(4μM), DnaJ(0,8 μM), GrpE(0,2μM), ClpB(2μM)	+ + + +	+ + + +	+ + + +	+ + + +
GroEL (2μM), GroES (2μM)	+ + + +	+ + + +	+ + + +	+ + + +

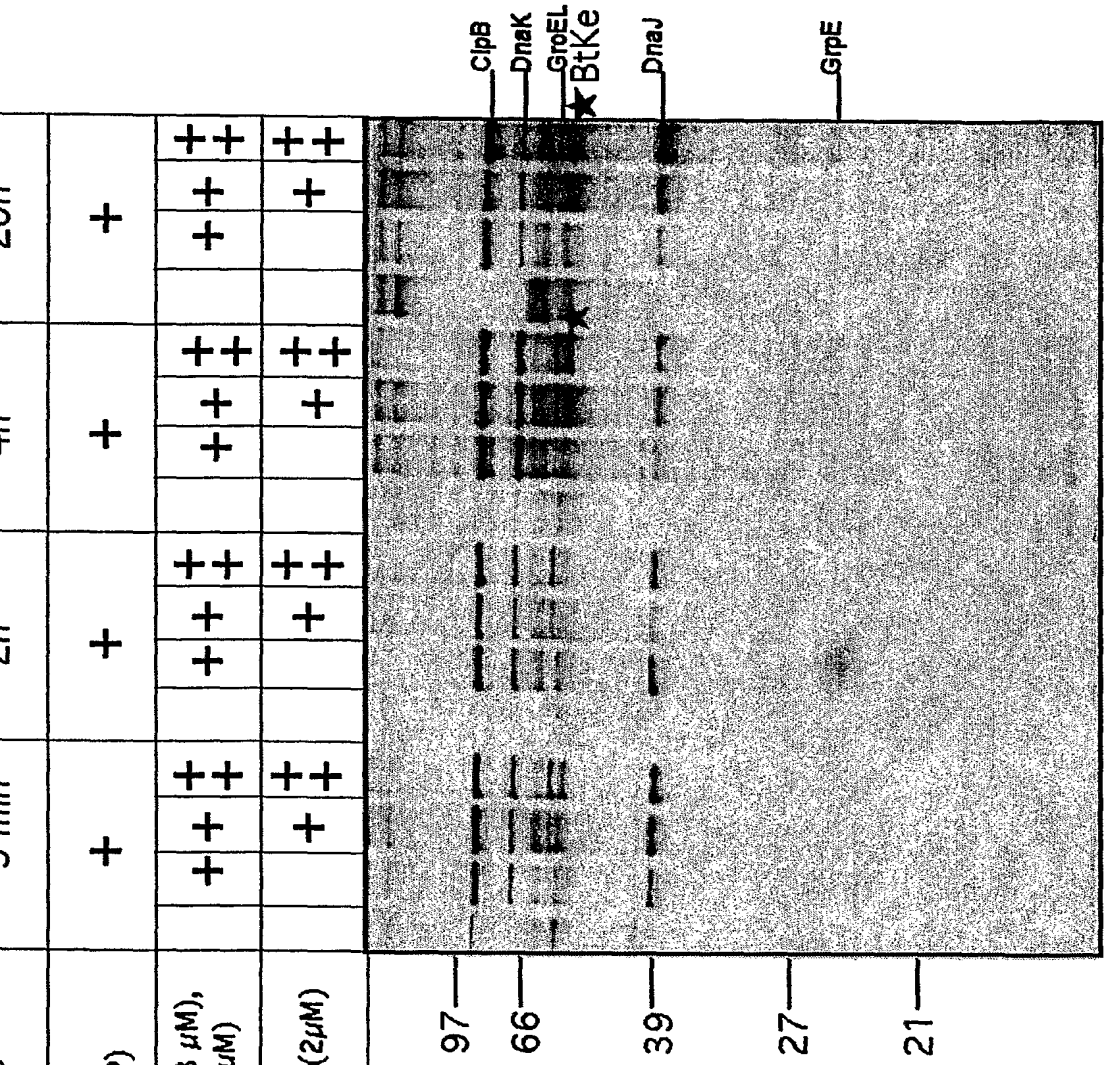


FIG. 5

MDH + 18.1 (pea)

↓ 30 min, 47°C

18.1/MDH

MDH + IbpB

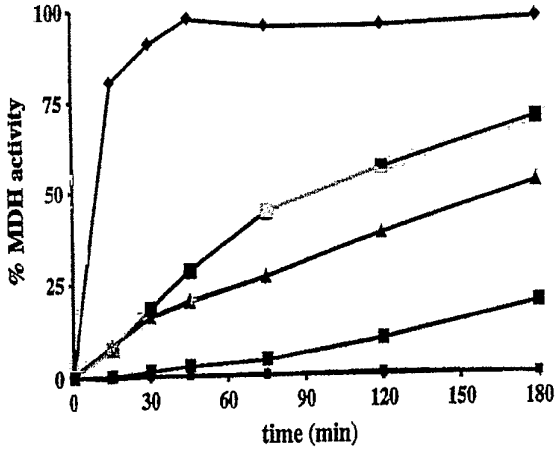
↓ 30 min, 47°C

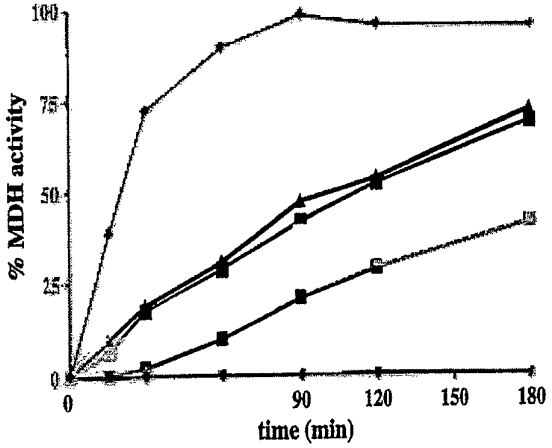
IbpB/MDH

MDH + 16.6 (*Synechocystis* sp.)

↓ 30 min, 47°C

16.6/MDH





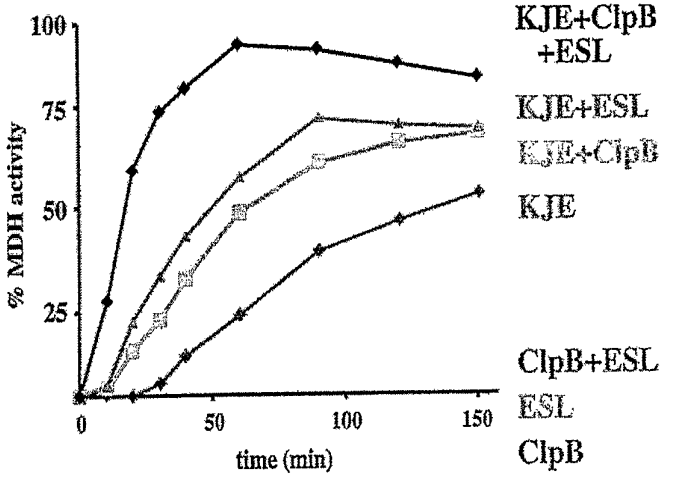


FIG. 6

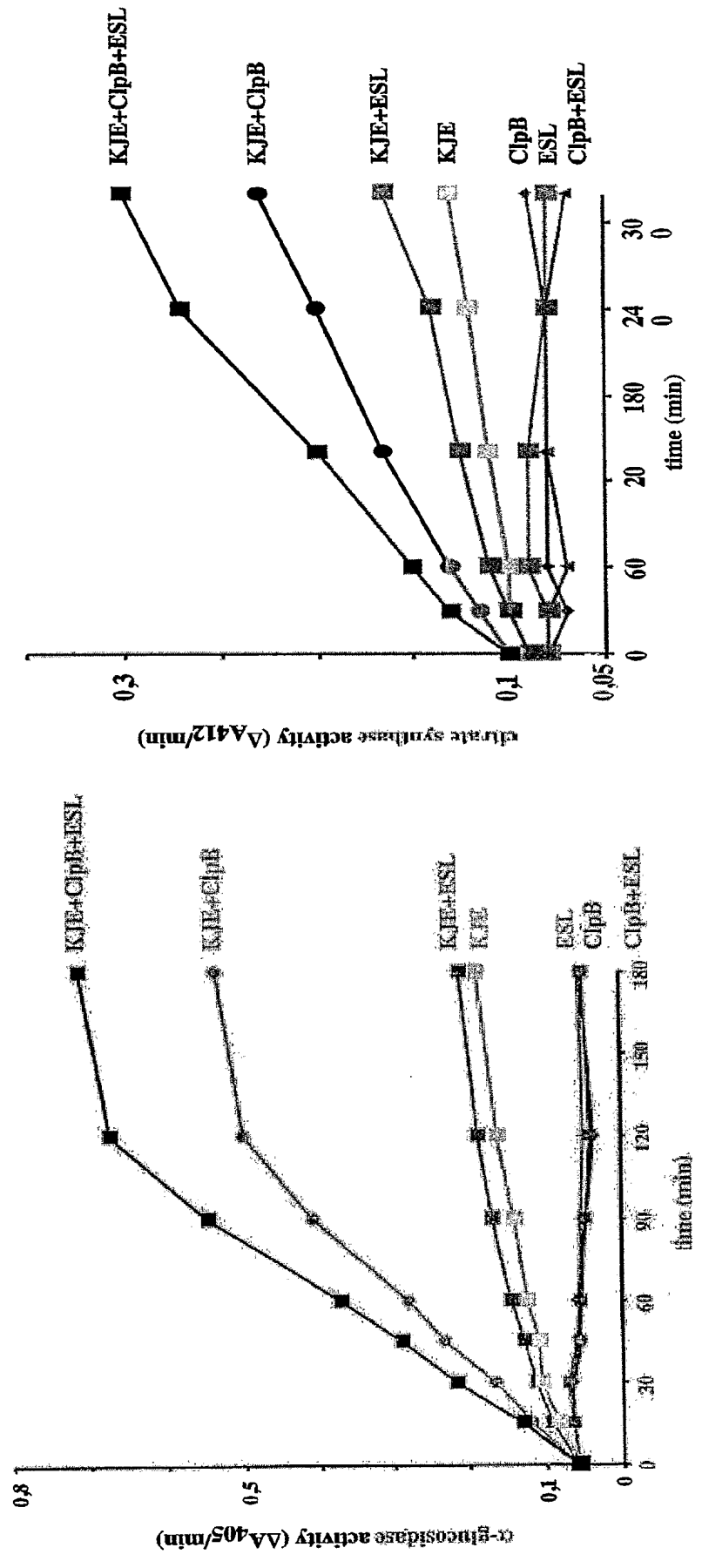

16.6/α-glucosidase complexes
α-glucosidase activity (ΔA405/min)
0,8
0,5
0,1
0
30
60
90
120
150
180
time (min)
KJE+ClpB+ESL
KJE+ClpB
KJE+ESL
KJE
ESL
ClpB
ClpB+ESL
16.6/citrate synthase complexes
citrate synthase activity (ΔA412/min)
0,3
0,1
0,05
0
60
20
180
24
0
30
0
time (min)
KJE+ClpB+ESL
KJE+ClpB
KJE+ESL
KJE
ClpB
ESL
ClpB+ESL

FIG. 7

Luciferase +/- 16.6 (sHsp), 15 min at 43°C

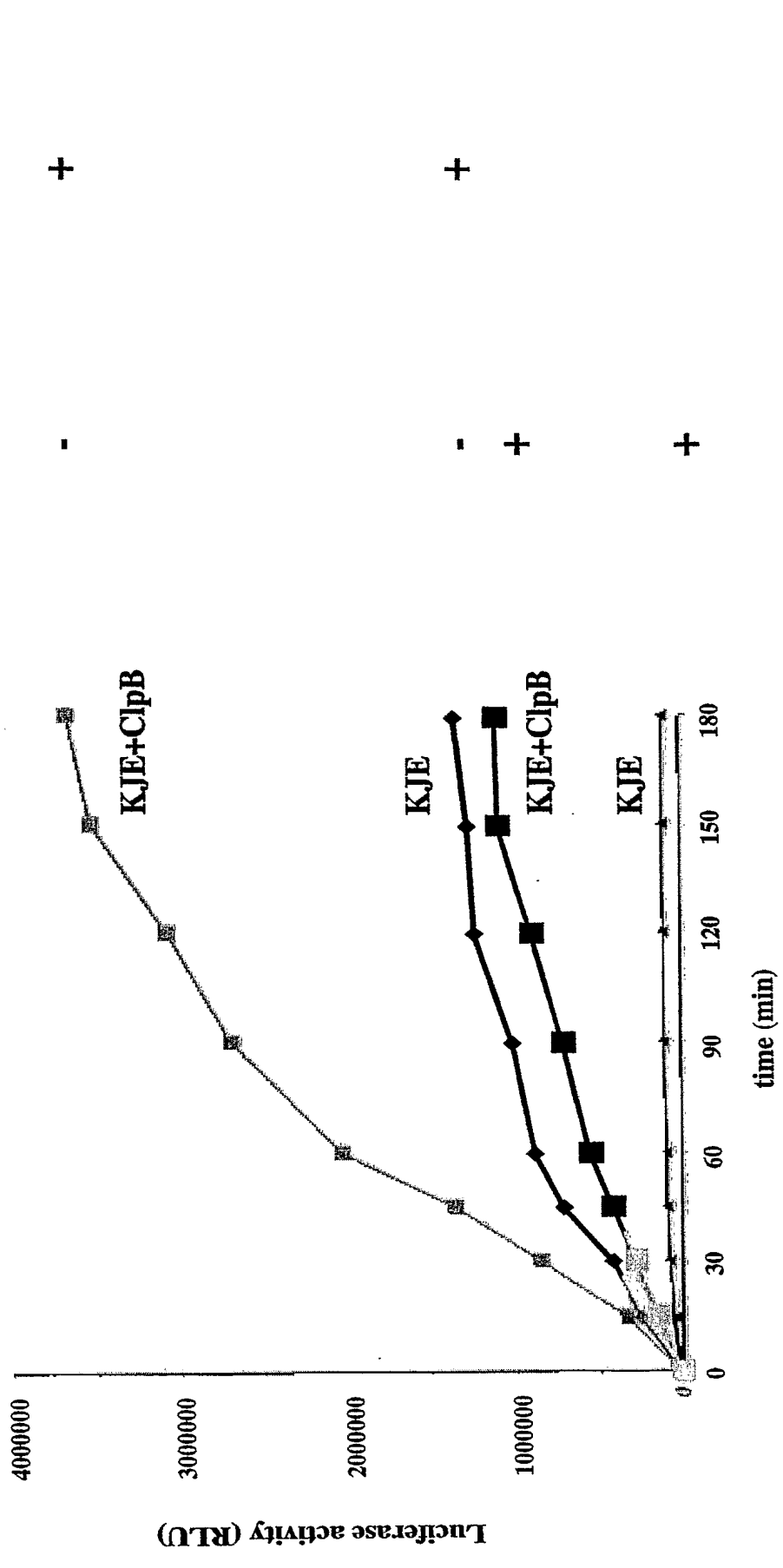


FIG. 8

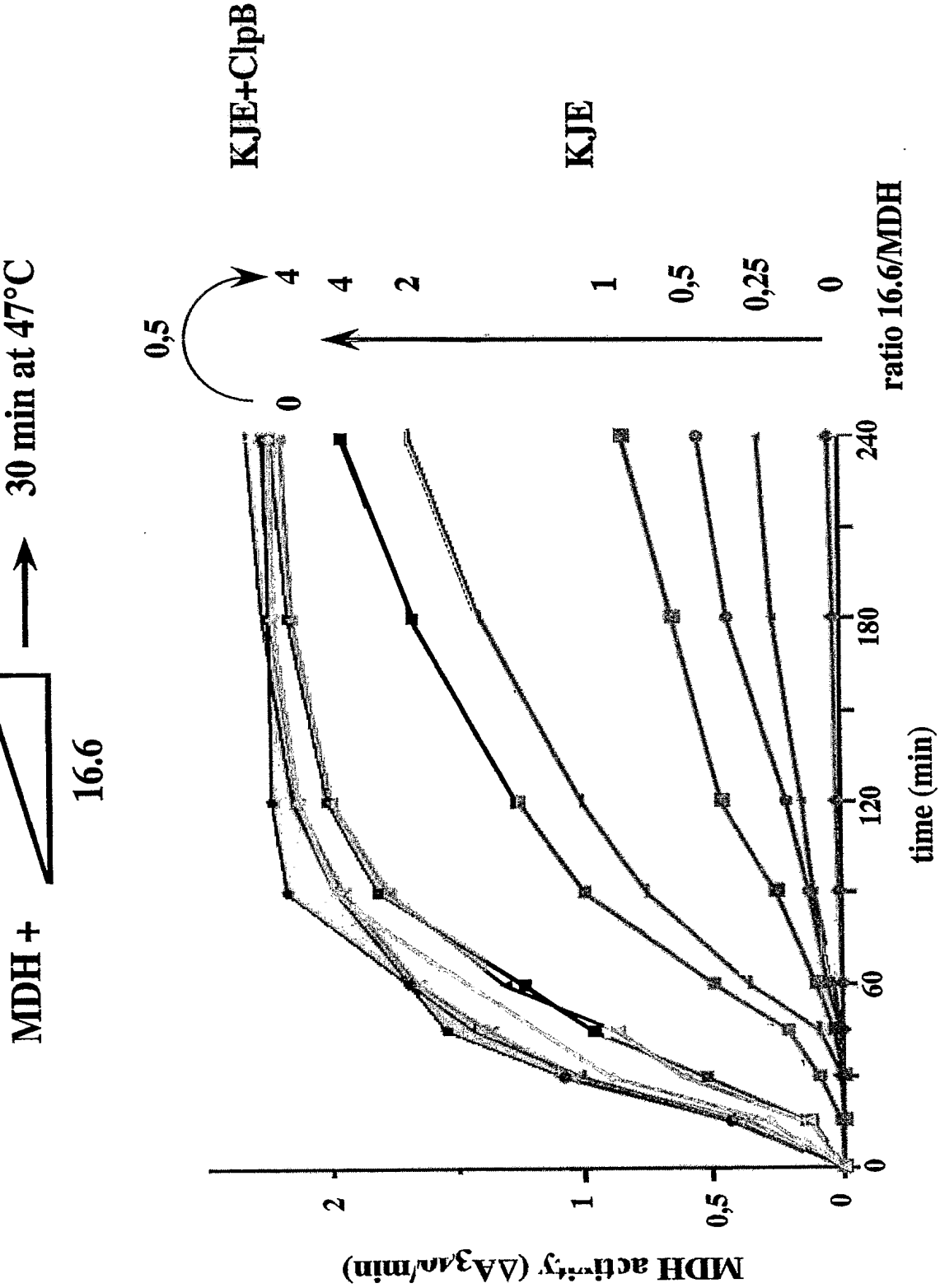

MDH +
16.6
30 min at 47°C
KJE+ClpB
KJE
0,5
0
4
4
2
1
0,5
0,25
0
ratio 16.6/MDH
MDH activity (ΔA340/min)
2
1
0,5
0
0
60
120
180
240
time (min)

FIG. 9

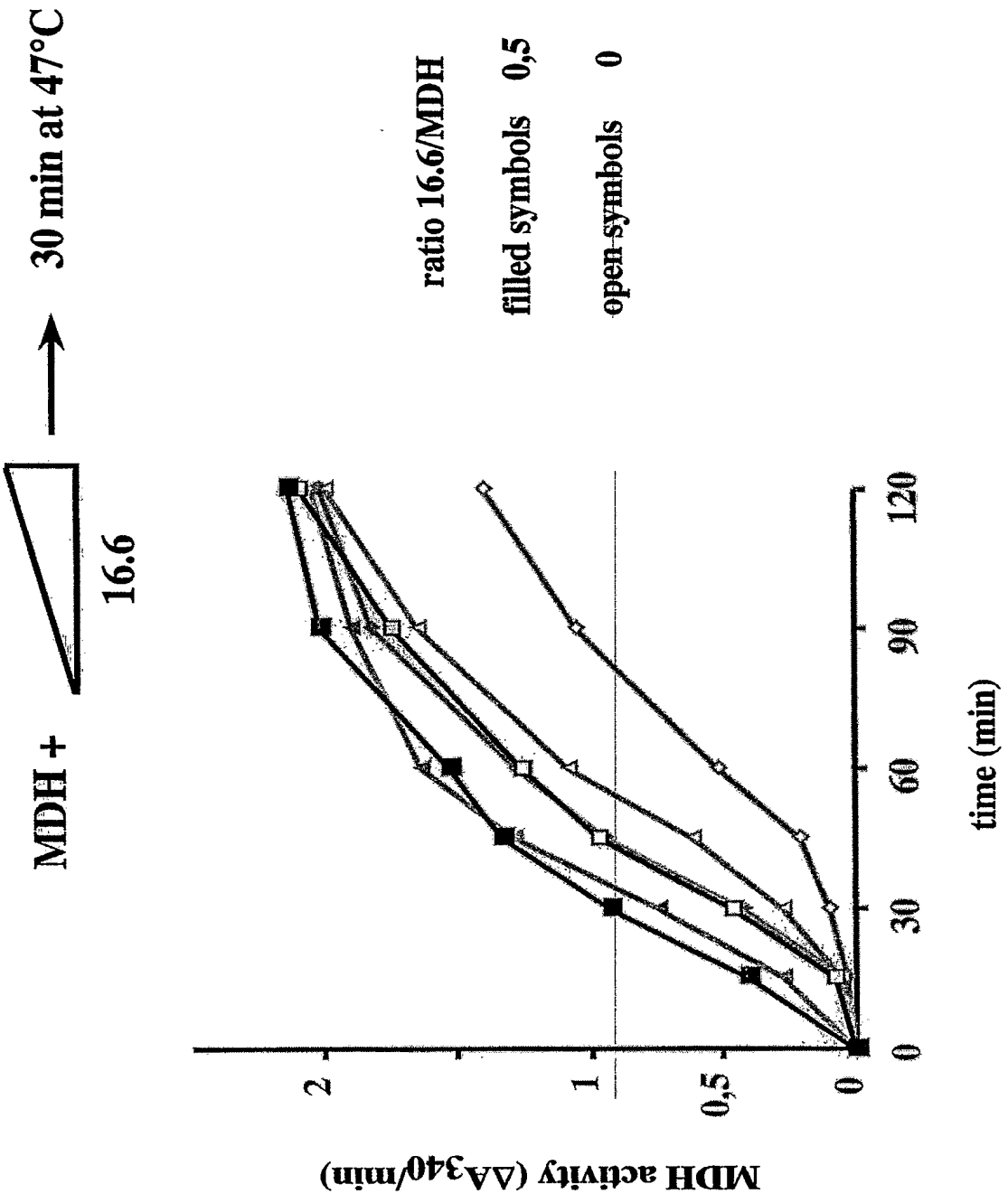

MDH +
16.6
30 min at 47°C
ratio 16.6/MDH
filled symbols 0,5
open symbols 0
ClpB conc. (µM)
1,5
0,5
0,15
MDH activity (ΔA340/min)
2
1
0,5
0
0
30
60
90
120
time (min)

FIG. 10

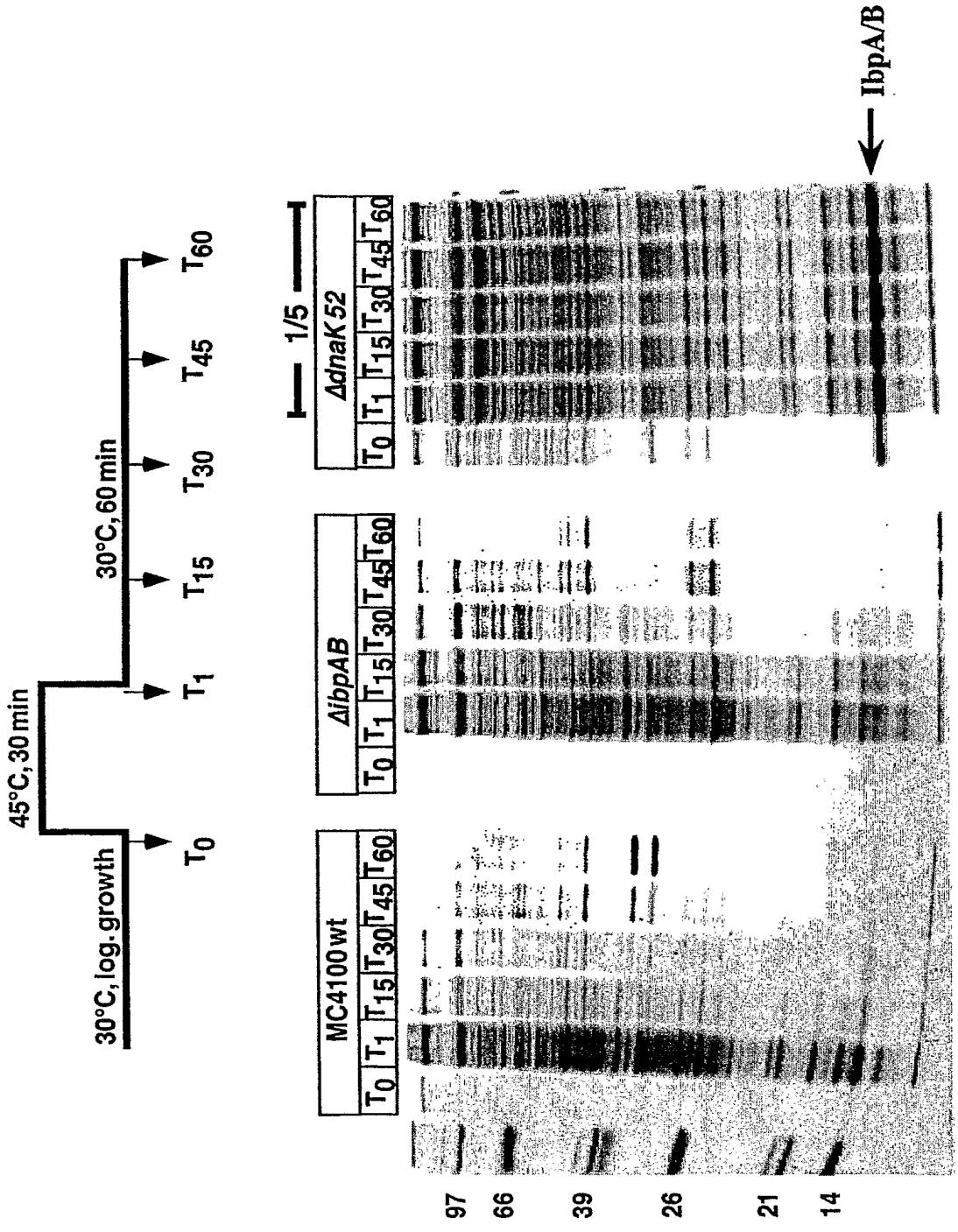

30°C, log. growth
45°C, 30 min
30°C, 60 min
T0
T1
T15
T30
T45
T60
MC4100 wt
ΔibpAB
ΔdnaK52
1/5
IbpA/B
97
66
39
26
21
14

FIG. 11

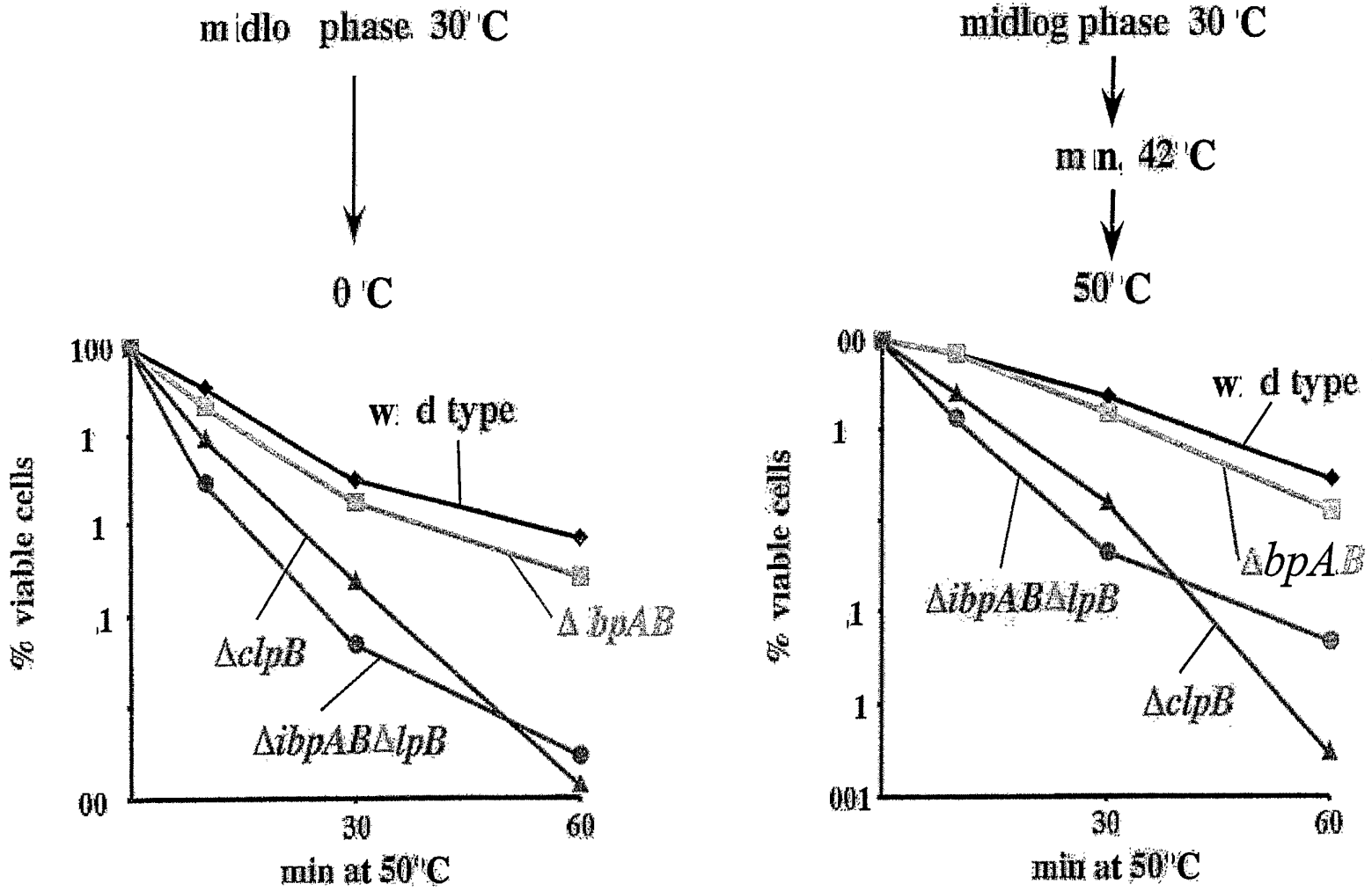

Thermotoerance is reduced in ΔibpAB and impaired ΔclpB mutan cell
midlo phase 30 C
0 C
100
1
1
.1
00
% viable cells
w d type
Δ bpAB
ΔclpB
ΔibpABΔlpB
30
60
min at 50 C
midlog phase 30 C
min, 42 C
50 C
00
1
.1
001
% viable cells
w d type
ΔbpAB
ΔibpABΔlpB
ΔclpB
30
60
min at 50 C

FIG. 12

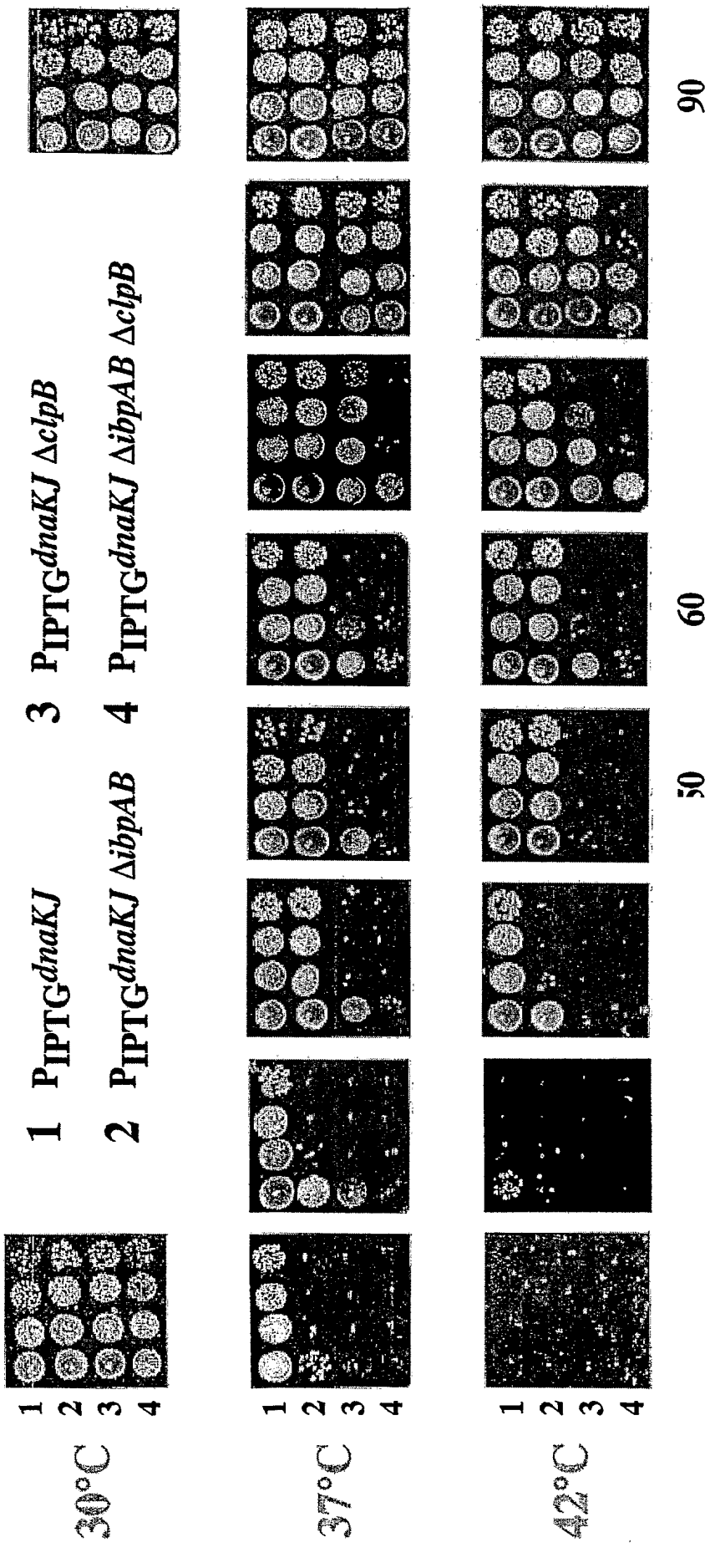

1 P_{IPTG}*dnaKJ*
2 P_{IPTG}*dnaKJ ΔibpAB*
3 P_{IPTG}*dnaKJ Δclpb*
4 P_{IPTG}*dnaKJ ΔibpAB Δclpb*
30°C
37°C
42°C
1 2 3 4
50
60
90
μM IPTG

FIG. 13

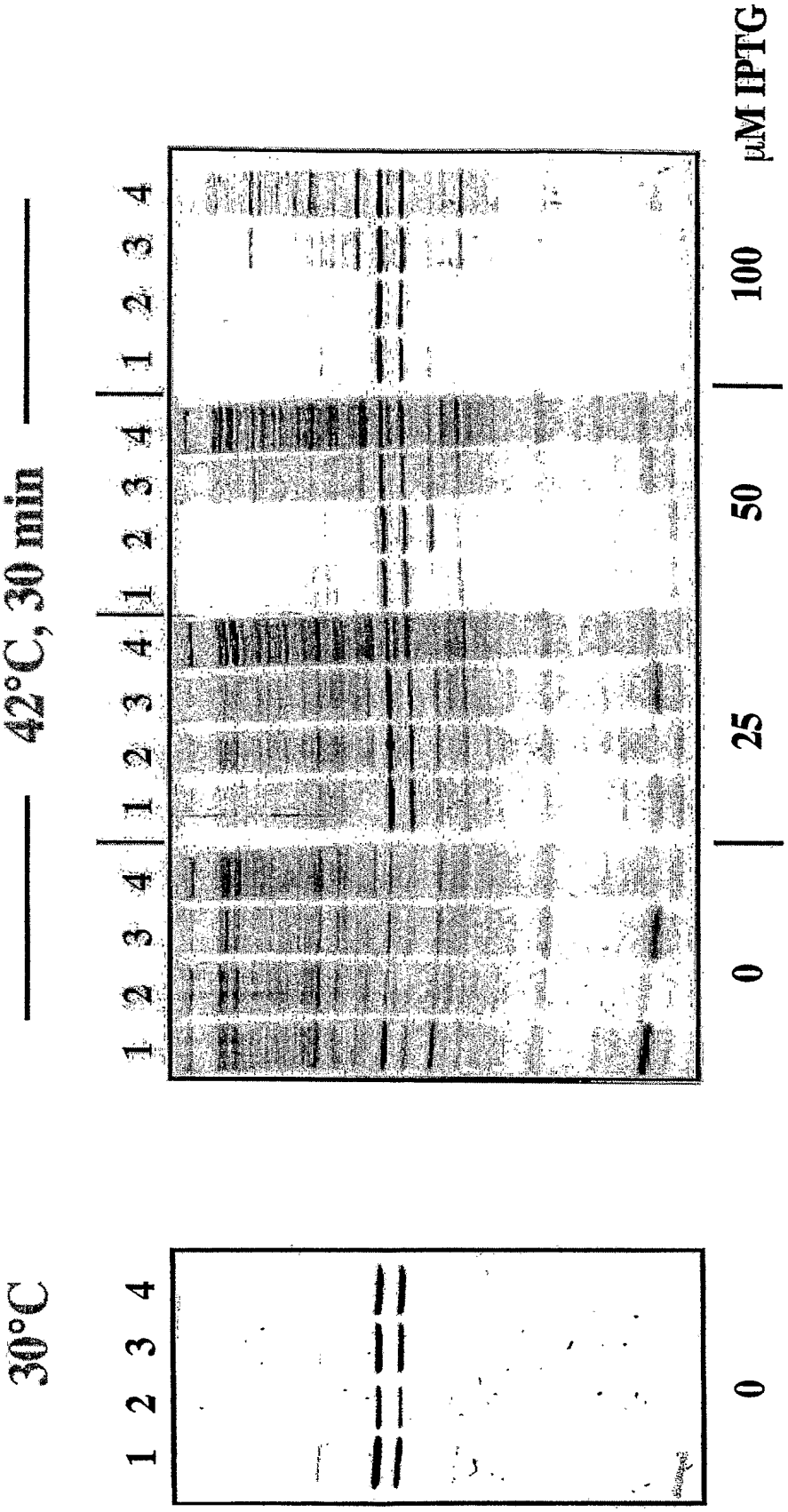

30°C
1 2 3 4
0
42°C, 30 min
1 2 3 4 1 2 3 4 1 2 3 4 1 2 3 4
0 25 50 100
μM IPTG
1 P_{IPTG}*dnaKJ*
2 P_{IPTG}*dnaKJ* Δ*ibpAB*
3 P_{IPTG}*dnaKJ* Δ*clpB*
4 P_{IPTG}*dnaKJ* Δ*ibpAB* Δ*clpB*

FIG. 14

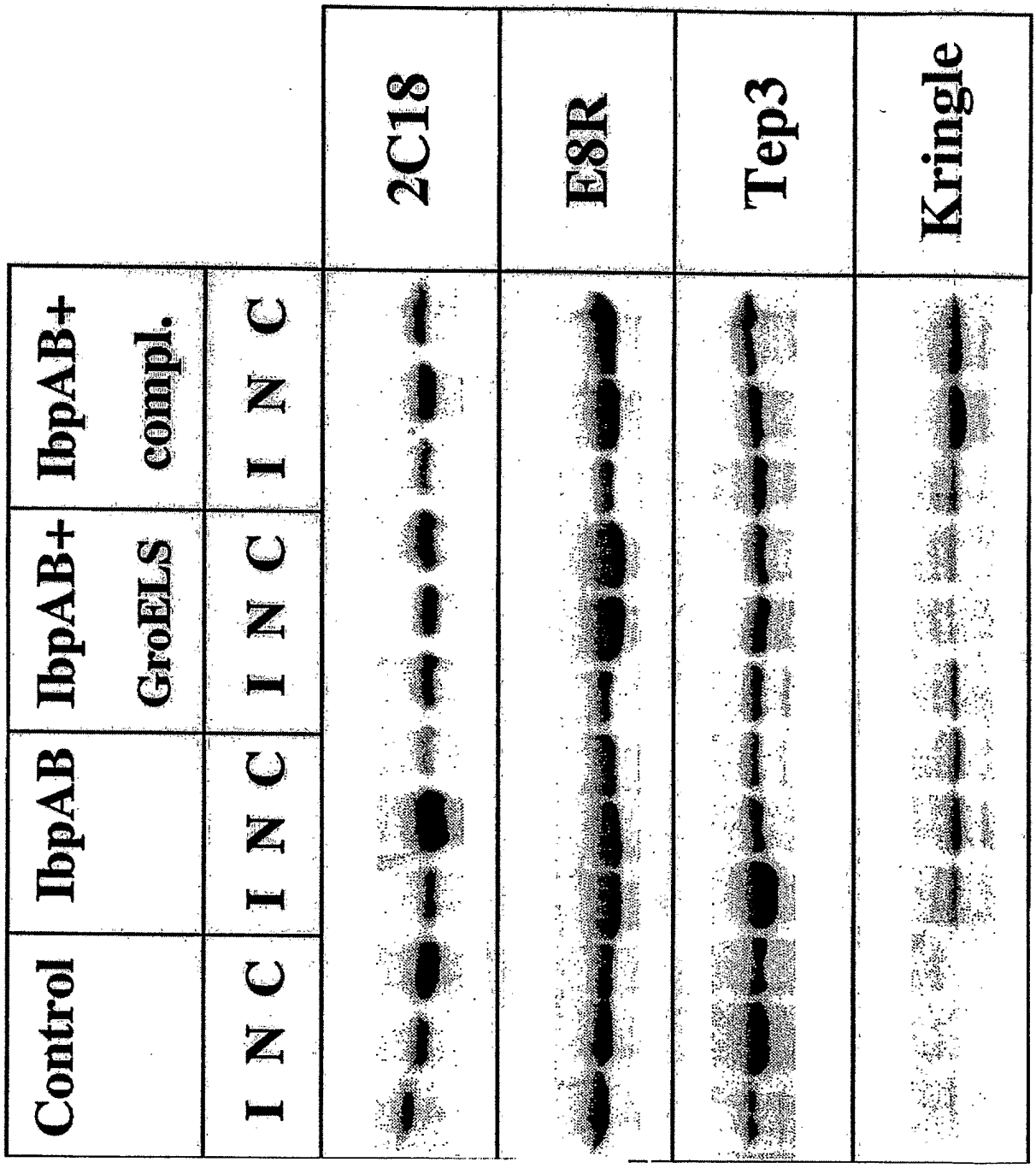
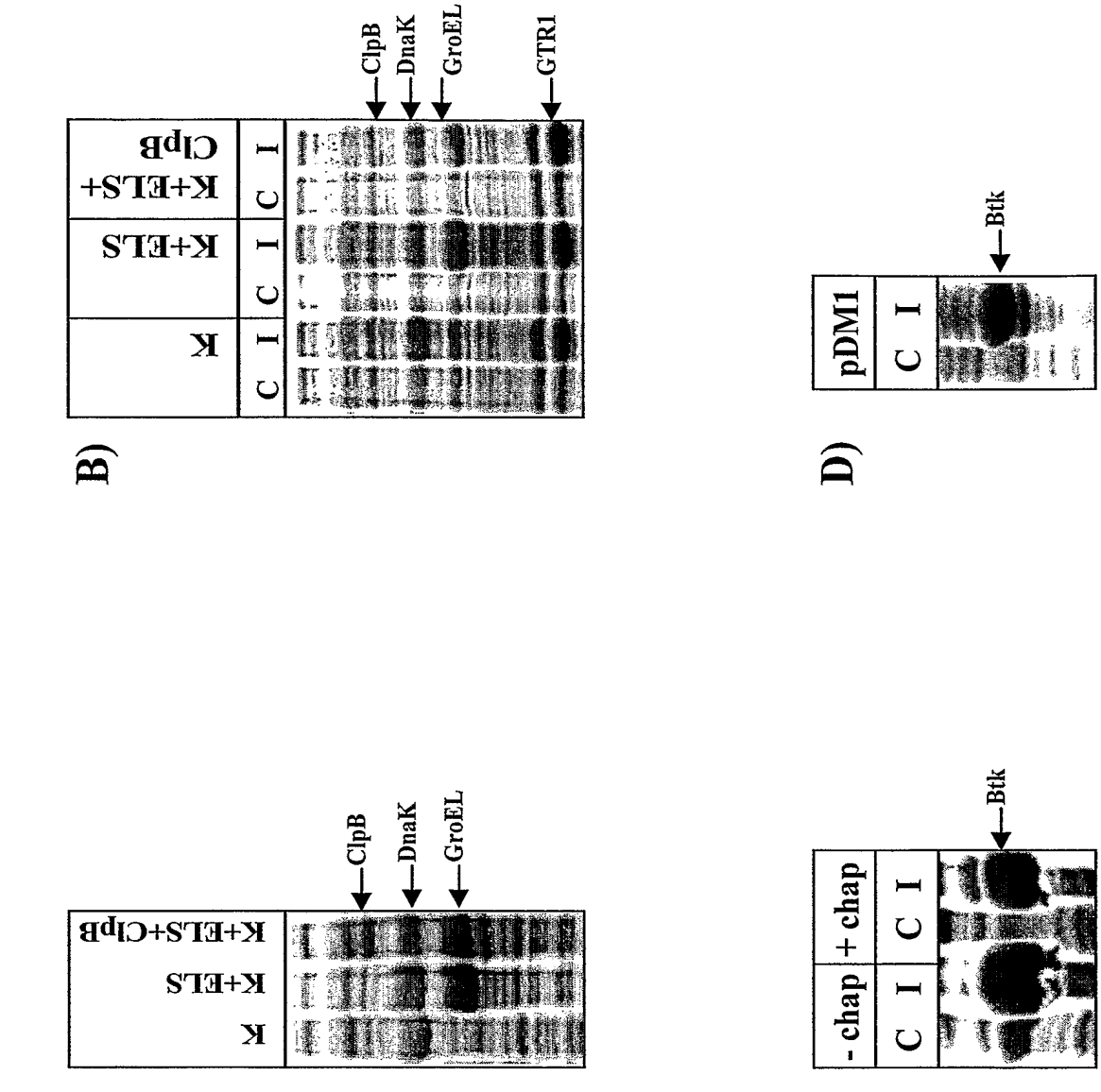

Control
IbpAB
IbpAB+ GroELS
IbpAB+ compl.
I N C I N C I N C I N C
2C18
E8R
Tep3
Kringle

Figure 15


A)
ClpB
DnaK
GroEL
K
K+ELS
K+ELS+ClpB
B)
ClpB
DnaK
GroEL
GTR1
K
K+ELS
K+ELS+ ClpB
C I C I C I C I
C)
Btk
- chap
+ chap
C I C I
D)
Btk
pDM1
C I

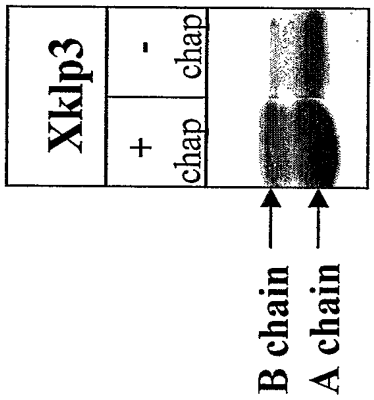
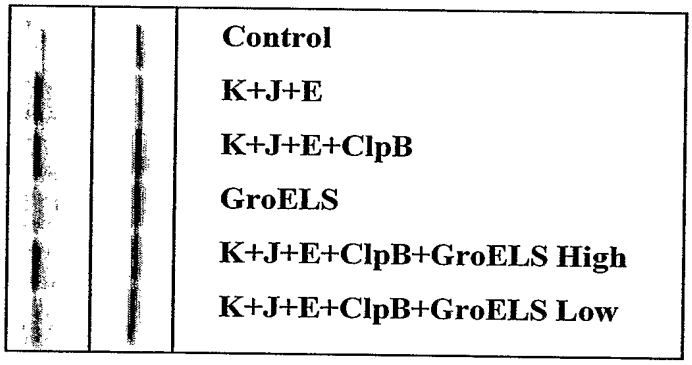


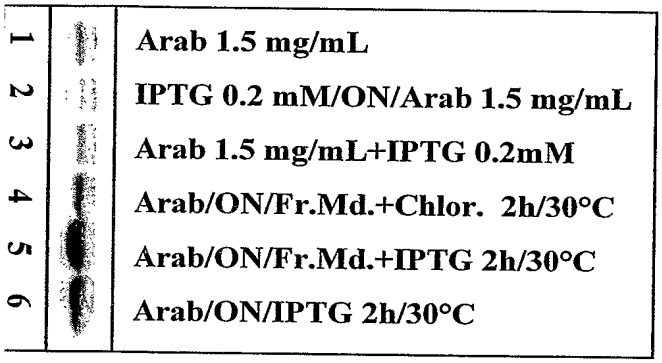
Figure 16

Figure 17

A)



C)



B)